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DISCOVERING A NOVEL ANTIFUNGAL TARGET IN DOWNSTREAM STEROL BIOSYNTHESIS USING A SQUALENE SYNTHASE FUNCTIONAL MOTIF

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DISCOVERING A NOVEL ANTIFUNGAL TARGET IN DOWNSTREAM
STEROL BIOSYNTHESIS USING A SQUALENE SYNTHASE FUNCTIONAL MOTIF

DISSERTATION

A dissertation submitted in partial fulfillment of the
requirements for the degree of Doctor of Philosophy in the
College of Medicine
at the University of Kentucky

By
Kristin Brooke Linscott

Lexington, Kentucky

Director: Dr. Joe Chappell, Professor of Pharmaceutical Sciences

Lexington, Kentucky

2017

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ABSTRACT OF DISSERTATION

DISCOVERING A NOVEL ANTIFUNGAL TARGET IN DOWNSTREAM STEROL BIOSYNTHESIS USING A SQUALENE SYNTHASE FUNCTIONAL MOTIF

The sterol biosynthetic pathway is essential for growth of all eukaryotic cells and the main target of antifungal agents. The emergence of resistance to these antifungals in an already ill patient population indicates a need to develop drugs that have a broad spectrum of activity among pathogenic fungi and have minimal patient toxicity. Squalene synthase is the first committed step in the sterol pathway and has been studied intensively for development of antifungal agents. While the overall architecture of this enzyme is identical throughout eukaryotes, it was shown that plant and animal genes cannot complement a squalene synthase knockout mutation in yeast unless the carboxy-terminal domain is swapped for one of fungal origin. This implies that there is a component of the fungal carboxy-terminal domain that is responsible for the complementation phenotype and that is unique to the fungal kingdom of life.

To determine the role of the carboxy-terminal domain of squalene synthase in the sterol pathway, we used the yeast *Saccharomyces cerevisiae* with a squalene synthase knockout mutation and expressed squalene synthases originating from fungi, plants, and animals. In contrast to previous observations, all enzymes tested could partially complement the knockout mutation when the genes were weakly expressed. When induced, non-fungal squalene synthases could not complement the knockout mutation and instead led to the accumulation of carboxysterol intermediates, suggesting an interaction between squalene synthase and the downstream sterol C4-decarboxylase. Overexpression of a sterol C4-decarboxylase from any kingdom of life both decreased the accumulation of carboxysterol intermediates and allowed non-fungal squalene synthases to complement the squalene synthase knockout mutation.

Using chimeric squalene synthases from each kingdom of life, the motif in the C-terminal domain responsible for preventing this toxicity was mapped to a kingdom-specific 26-amino acid hinge motif adjacent to the catalytic domain. Furthermore, over-expression of the carboxy-terminal domain alone containing a hinge motif from fungi, not from animals or plants, led to growth inhibition of wild-type yeast. Since this hinge region is unique to and highly conserved within each kingdom of life, this data provides evidence for the development of an antifungal therapeutic as well as for tools to develop an understanding of triterpene catalytic activity and identify similar motifs in other biosynthetic pathways.

KEYWORDS: Squalene Synthase, Kingdom-specific Motif, Genetic Complementation
Sterol C4-decarboxylase, Growth Inhibition

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STEROL BIOSYNTHESIS USING A SQUALENE SYNTHASE FUNCTIONAL MOTIF

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To my father, for every pearl of wisdom
and cup of coffee

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Chapter 1: Background and Introduction

The Emergence of Invasive Fungal Infections

While invasive fungal infections were once rare in a clinical setting, their emergence is now an important cause of patient morbidity and mortality [1]. For many years, invasive fungal diseases (involving blood, deep tissues, and organs) were caused predominantly by endemic fungi. These fungi, such as *Histoplasma capsulatum*, *Blastomyces dermatitidis*, and *Coccidioides immitis/posadasii*, were found in a particular geographic area due to local adaptation and host specificity [2–5]. Infections began in the lungs following inhalation of spores and then quickly progressed to deep viscera even in healthy individuals [6]. While these endemic fungi continue to infect patients, the epidemiology associated with invasive fungal infections has altered over the past few decades and now predominantly identifies opportunistic fungi in the expanding population of at-risk hospitalized patients. Despite a growing body of evidence and knowledge about these fungi, the diagnosis and management of invasive fungal infections remains a challenge.

Opportunistic infections specifically infect immunocompromised individuals. Of the more than 18 different genera of opportunistic fungi that infect humans today, *Candida* species such as *C. albicans*, *C. glabrata*, *C. krusei* and *C. parapsilosis* represent the most common causative agents, accounting for between 46% and 53% of all reported cases. They are also the most often isolated fungi in cases where the patients have solid tumors. *Aspergillus* species, such as *A. fumigatus*, make up the second most common cause of invasive fungal infections (18%-24.8%) and are the most often associated with organ transplantation, particularly after hematopoietic stem cell transplantation [1,7] (Fig. 1.1A). Opportunistic infections also include the less common fungi that are the cause of cryptococcosis and zygomycosis.

The association of fungal infections with healthcare is a common one since opportunistic infections are often caused by improved medical technologies which increase the survival of patients with conditions that render them susceptible to infection. For instance, patients with cancer are often at an increased risk of infection during chemotherapy, and many develop a lifelong susceptibility to certain infections even after cancer treatment has ended. Improved intensive care units treat patients with high doses of powerful antimicrobial drugs,

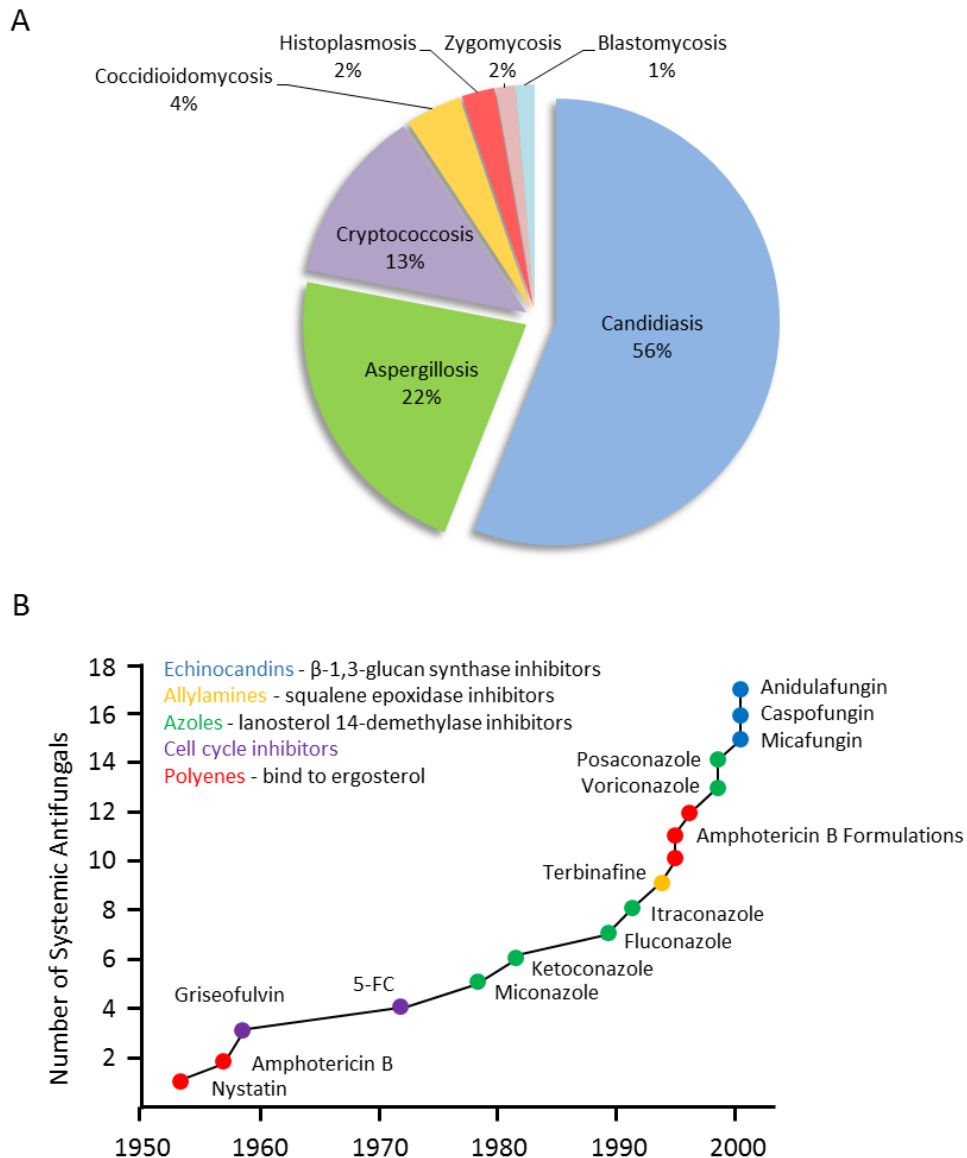


Figure 1.1: *Candida* and *Aspergillus* are the most common fungi associated with invasive fungal infections in humans and there are few available treatment options. (A) Of the 7 most common genera of fungi that cause invasive infections in humans, *Candida* (56%) and *Aspergillus* (22%) are the most common. (B) The number and rate of development for available classes of systemic antifungal agents introduced to the clinic from 1950-2000 are listed with polyenes (red), cell cycle inhibitors (purple), azoles (green), allylamines (yellow), and echinocandins (blue). This figure is modified from Menzin *et al.* (2009) and Ostrosky-Zeichner *et al.* (2010).

decreasing competition between fungi and other microbes, and organ transplants often necessitate long-term use of immunosuppressive agents. In these patients, even a small primary infection may progress to fulminant, disseminated disease if not properly treated [8].

It is not surprising then that the frequency of invasive fungal infections due to these opportunistic pathogens has increased. Hospitalizations caused by fungal infections rose by approximately 5.7% per year throughout the 1980s and early 1990s. For *Aspergillus* alone, the increase was at least eightfold over this period of time [9]. Studies have shown that the number of cases of sepsis caused by fungi increased by 207% in the twenty years leading up to the turn of the century [10]. While there are only a few recent epidemiological reports concerning the incidence of invasive fungal infections, a review of these reports has suggested that more people worldwide die from the ten most prevalent invasive fungal diseases than from tuberculosis or malaria [11]. In one of the most complete studies of nosocomial bloodstream infections, the Surveillance and Control of Pathogens of Epidemiological Importance study (SCOPE) revealed that almost 10% of all infections were caused by fungi, and *C. albicans* was the fourth leading cause of blood stream infections [12].

Perhaps the most striking finding is that the mortality rate for invasive fungal infections has not dramatically improved. A nationwide study in France in 2014 found that among the 3,600 patients who developed invasive fungal diseases, 28% died of complications arising from the infection [1]. Of the estimated 400,000 cases of candidemia that occur annually, the mortality rate is reported to be 42%, while the estimated 200,000 cases per year of invasive aspergillosis have a mortality rate that is consistently reported to be greater than 50%, and as high as 75-80% even if the patients are properly diagnosed and treated [11].

The Development of Antifungal Therapeutics

The steadily increasing incidence of fungal diseases and disappointingly high mortality rates argue for a change in the way that we treat these infections. Currently, one of the most limiting factors in clinical care of invasive fungal infections is diagnosis, as many of the current antifungal agents do not treat a broad spectrum of fungi. Even when the causative agent has been identified, there are relatively few families of antifungal agents that treat systemic infections (Fig. 1.1B). Compared to other pathogens, fungi are evolutionarily close to humans, and this has a limiting effect on drug discovery and development. For this reason, therapeutic

targets often involve key steps in cell wall and membrane development, especially on the fungal membrane sterol, ergosterol, and its biosynthesis.

Of the antifungal agents available to clinics, three major classes inhibit the ergosterol biosynthetic pathway: allylamines, azoles, and polyenes. The allylamine, terbinafine, inhibits the enzyme squalene epoxidase (Erg1) by binding to the enzyme and triggering a conformational change that prevents the substrate from binding [13]. While these drugs are fungicidal in susceptible species including many filamentous fungi, they are not capable of inhibiting the growth of most pathogenic yeast [14,15]. Azoles, such as fluconazole, inhibit the enzyme lanosterol C14-demethylase. Unlike allylamines, the azoles are fungistatic and so require a functional immune system to remove the growth inhibited fungi. Moreover, resistance to azoles is currently rising due not only to its common use in clinics but also to its use as an antifungal for crop management [16]. The polyenes are a class of compounds that bind to ergosterol and disrupt membrane permeability. These toxic agents may cause severe and sometimes lethal multiple organ failure [17]. One other class of antifungal agents that is commonly used to target invasive fungal infections in clinics is the echinocandins, which target the enzyme complex responsible for synthesizing beta-(1,3)-glucan in the cell wall [18]. Unlike other antifungal agents, echinocandins have fungicidal activity against *Candida* species, even in critically ill patients.. However, clinicians are now observing an increase in fungi that are resistant to both azoles and echinocandins [19].

Over the past decade, research has turned its focus toward other biosynthetic pathways and cellular components as potential targets for fungal growth inhibition, including amino acid and sphingolipid biosynthesis [20]. These drugs, while active against many varieties of pathogenic yeast, are not as active against pathogenic filamentous fungi like *Aspergillus*, and many are limited by toxicity. Results like these, while they point to promising lead structures, have not yielded compounds with the desired potency, spectrum of activity, or safety necessary for clinical use. These circumstances led to the studies reported in this thesis in which we sought to identify other potential therapeutic targets in the fungal sterol biosynthetic pathway.

Squalene Synthase Structure and Function

The enzyme squalene synthase (SQS) has garnered attention in the research community for its role as the first committed step in the sterol pathway – a gatekeeper determining how carbon flux is distributed between sterols and other essential isoprenoids (6). Initial interest in

using this enzyme as a drug target focused on the fact that it is downstream from HMG-CoA reductase in the cholesterol biosynthetic pathway. Therefore, inhibition of sterol biosynthesis at this step would avoid a decrease in ubiquinone thought to be responsible for the development of myopathy, while still providing a similar mechanism of action to statins [21,22]. While several inhibitors of squalene synthase have undergone early development as potential lipid-altering drugs, they have been associated with potential hepatic toxicity and have not completed clinical trials [23]. The viability of these inhibitors for decreasing cholesterol biosynthesis in humans is still questionable, but the use of SQS inhibitors is now widely studied for the production of anti-fungal therapeutics. This enzyme is also believed to be a promising target for inhibiting growth of the protozoan parasites *Leishmania donovani* and *Trypanosoma cruzi* [20,24,25], and for disrupting virulence factor biosynthesis in the bacteria *Staphylococcus aureus* [26].

Development of squalene synthase inhibitors is limited by the fact that the active site, in fact the overall architecture of this enzyme, is highly conserved throughout the eukaryotic kingdoms of life. SQS consists of two domains: the amino (N)-terminal catalytic domain and a carboxy (C)-terminal domain responsible for tethering the enzyme to the cytosolic face of the endoplasmic reticulum (ER) (Fig. 1.2A). Within the catalytic domain, alignment of the amino acid sequences from each of these SQS proteins revealed highly conserved sequences (domains I-V) responsible for the catalytic reactions performed by this enzyme (Fig. 1.3) [27]. First, head-to-head dimerization of two molecules of farnesyl diphosphate (FPP) forms the intermediate presqualene diphosphate (PSPP) that contains a cyclo-propane ring. The ring is cleaved followed by reductive rearrangement with the co-factor NADPH to form squalene [28] (Fig. 1.2B). Squalene then proceeds into the sterol pathway where it is further activated by squalene epoxidase, then cyclized and modified to form ergosterol in fungi (Fig. 1.4A), cholesterol in mammalian cells, and the three major sterols in plants; stigmasterol, sitosterol, and campesterol [29]. The C-terminal membrane spanning helix is critical for localizing SQS to the ER membrane, where it is part of a complex network of protein-protein interactions involving the rest of the steps in this pathway. Mo and Bard used a membrane-based yeast two-hybrid system to characterize interactions between all of the sterol biosynthetic proteins in *S. cerevisiae* and suggested that disruptions in this “ergome” may delocalize or destabilize other proteins impacting the efficiency of the pathway (Fig. 1.4B).

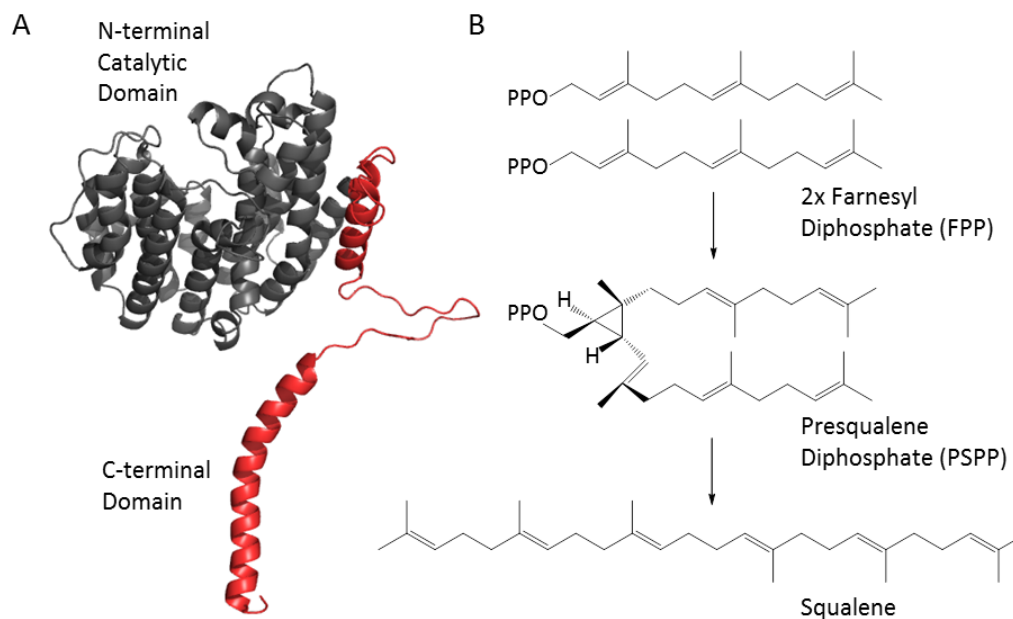


Figure 1.2: Squalene synthase architecture and catalytic activity. (A) A homology model of yeast SQS. This enzyme is composed of both a helical amino- terminal catalytic domain and a carboxy-terminal domain which includes a membrane spanning helix responsible for tethering the enzyme to the cytosolic face of the ER membrane. (B) SQS performs a reductive dimerization, converting two molecules of farnesyl diphosphate (FPP) to squalene through a two-step mechanism within a single active site cavity.

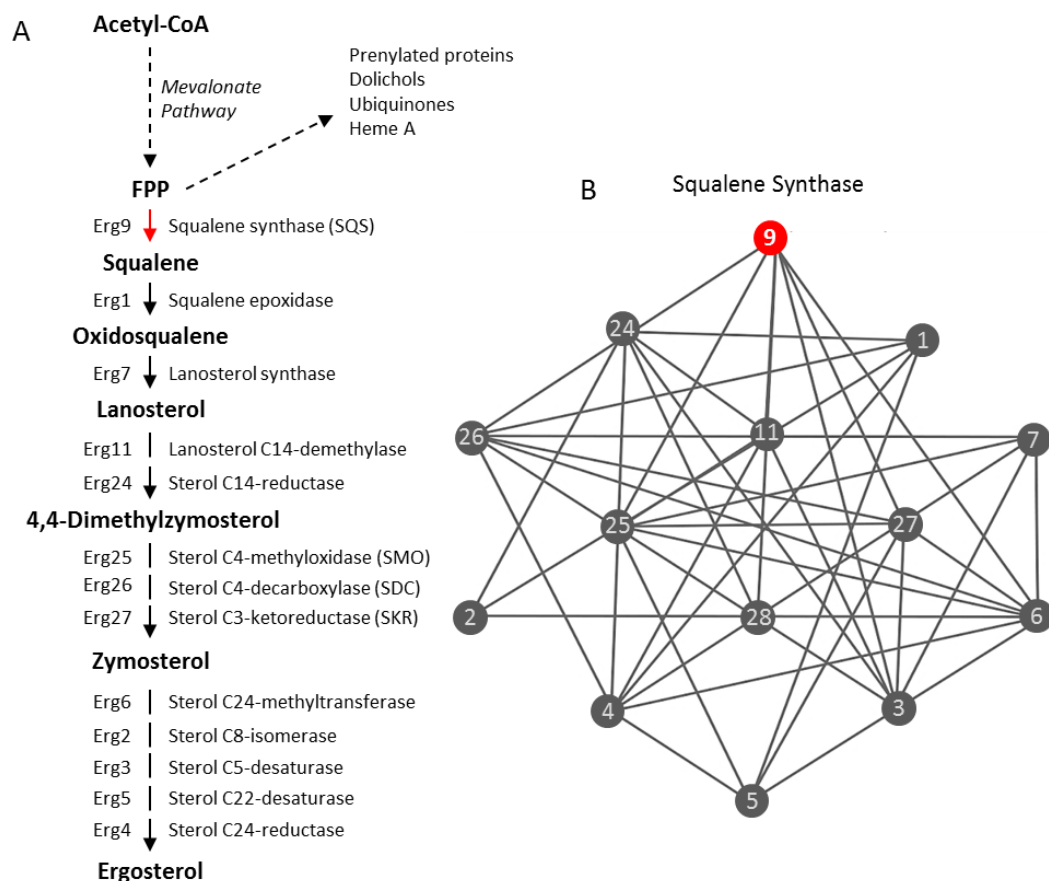


Figure 1.4: The sterol biosynthetic pathway in fungi and the sterol biosynthetic complex. (A) SQS is the first step (red arrow) in the ergosterol biosynthetic pathway converting farnesyl diphosphate (FPP) to squalene, a 30-carbon isoprenoid oxidized by squalene monooxygenase (Erg1) and cyclized by lanosterol synthase (Erg7). In *S. cerevisiae*, ten additional enzymes modify the position of methyl groups and double bonds to form ergosterol. Dashed lines indicate multiple steps in the pathway. (B) These enzymes are all believed to be interconnected in a protein complex. Each circle represents an enzyme in the *S. cerevisiae* sterol biosynthetic pathway, with lines depicting interactions identified by yeast 2-hybrid analysis. SQS (Erg9) is colored red. This figure is modified from Mo and Bard (2005).

Scope of Dissertation

Considering the high morbidity and mortality associated with emerging invasive fungal infections as well as the limited range of antifungal agents available in clinics, there is a need for antifungal agents with novel biochemical targets. The sterol biosynthetic pathway is a familiar but important target for the treatment of these infections, and the role of SQS as the first step in this pathway makes it an attractive option for drug development. As previously discussed, the C-terminal domain of SQS is responsible for tethering the enzyme to the endoplasmic reticulum, but this domain has another fungal-specific function. If a SQS is composed of a fungal C-terminal domain, not plant or animal, then it is able to complement a *SQS* knockout mutation in *S. cerevisiae* regardless of the origin of the catalytic domain. And so, a small molecule or peptidomimetic aimed at disrupting this function has the potential to be a safe and specific inhibitor of fungal growth in humans with broad spectrum efficacy among pathogenic fungi.

In order to gain a clearer understanding of the SQS C-terminal domain, this dissertation aimed to characterize this region of SQS in terms of yeast growth and determine how it may be used for antifungal development. Chapter 3 describes the phenotype of yeast expressing *SQS* genes from each different kingdom of life (fungal, plant, and animal) at both un-induced and induced levels and identifies a “toxicity” effect caused by over-expression of a non-fungal SQS. Chapter 4 investigates the role of SQS in the downstream sterol pathway and its effect on the activity of the C4 demethylation complex. Chapter 5 maps a kingdom-specific 26-amino acid hinge motif in the C-terminal domain of SQS responsible for the growth phenotypes described in the previous chapters and verifies its potential use for the development of a fungal growth inhibitor. Finally, we use the tools and knowledge gained through this investigation in the concluding chapter (Chapter 6) to develop a screen capable of evaluating active site residues of triterpene synthases and produce a preliminary algorithm for identifying other proteins with similar kingdom-specific motifs.

Chapter 2: Materials and Methods

Yeast Lines and Growth Media

The yeast line ZX178-08 was produced by Zhuang and Chappell [30] from the parental line BY4741 (MATa;his3 Δ 1;leu2 Δ 0;met15 Δ 0;ura3 Δ 0). In short, cells were mutagenized by EMS and plated on media containing nystatin, squalastatin, and cholesterol to select for colonies with a dispensable sterol pathway. The ZX178 yeast line was then selected from this cohort for its ability to grow only in the presence of exogenous ergosterol and accumulate high levels of farnesyl diphosphate (FPP). A targeted knockout mutation of the *SQS* gene was then performed by replacing the *ERG9* gene with the hygromycin resistance gene *hphNT1* [31]. Yeast line ZX178-08 was selected for its ability to grow on complete media with added ergosterol and 300 mg/L hygromycin B.

Additional targeted knockout mutations for yeast lines *Δ erg9,1* (ZXB) and *Δ erg9,7* (ZXE) were performed by Zhuang and Chappell using ZX178-08 [30]. In the same manner, the double knockout yeast lines *Δ erg9,6* and *Δ erg9,3* were produced using ZX178-08 as the parental line. The *ERG6* and *ERG3* genes were amplified using Takara high fidelity PrimeStar taq polymerase from yeast BY4741 genomic DNA. The PCR fragment was purified, A tailed and ligated into the pGEM Teasy vector (Promega). This construct was then used as a template for a second PCR reaction to obtain PCR fragments composed of only the 5' and 3' ends of the genes of interest to be used for homologous recombination. Two cloning sites were then inserted into the middle of these two sequences and the Padh-Kanmx4-Tcyc-LoxP antibiotic selection marker cassette was inserted between these restriction sites.

Yeast were grown without selection on YPD complete media with 1% Bacto-yeast extract, 2% Bacto-peptone, and 2% glucose (pH 5.3) or on synthetic complete (SC) media with 0.67% yeast nitrogen base without amino acids (-his, -leu, -ura, and -trp) (Difco), appropriate amino acids for selection (250 mg/L histidine, 1 g/L leucine, 300 mg/L uracil, 150 mg/L tryptophan), 0.6% succinic acid and either 2% glucose or 2% galactose (pH 5.5). YPDE and SCE media were supplemented with ergosterol for growth of ergosterol dependent lines. Solid YPDE and SCE media contained 4 mL of sterol stock solution (10 mg/mL ergosterol dissolved in Triton X-100 and ethanol 1:1) per liter while liquid YPDE and SCE media contained 0.5 mL of ergosterol stock solution per liter. Stock solutions of lanosterol or cholesterol were made in a manner identical to ergosterol. All solid media was prepared with 2% Bacto-agar.

Cloning Galactose-inducible Constructs

SQS genes (*ERG9*) from the yeast *S. cerevisiae* (X59959), *A. nidulans* (XM_655888), and *C. albicans* (D89610.1), the plants *A. thaliana* (NM_119630) and *N. benthamiana* (U46000.1), and the algae *B. braunii* (AF205791.1) were obtained from plasmids provided by Niehaus et al. [32]. The *H. sapiens* *ERG9* gene (FDFT1, NM_004462) was obtained through the Harvard Medical Center's DNA Resource CORE and was deposited by the NIH Mammalian Gene Collection (HsCD00331324). All *SQS* genes were cloned into the Pesc-ura vector (Agilent) using NotI and SpeI restriction sites.

The genes encoding for enzymes in the C4 demethylation complex of *S. cerevisiae*, sterol C4-methyloxidase (*ERG25*, NM_001181189.3), sterol C4-decarboxylase (*ERG26*, NM_001180866.1), and the sterol C3-ketoreductase (*ERG27*, NM_001181987.1), were amplified from BY4741 genomic DNA isolated using the YeaStar Genomic DNA Kit (Zymo Research). To identify an algal sterol C4-decarboxylase gene (*ERG26*, *SDC*), transcriptomic sequencing was performed by Niehaus et al using RNA pooled from *B. braunii* cultures ranging from 1-4 weeks after subculturing [33]. The DNA sequence data obtained by two transcriptomic profiling efforts was assembled with the CLC Genomics Workbench (CLC Bio). Screening of this dataset with the *S. cerevisiae* *Erg26* amino acid sequence revealed an ORF encoding a 354 amino acid protein with a predicted molecular weight of 38,270 daltons. The sterol C4-decarboxylase genes from *B. braunii*, *A. thaliana* (3 β HSD/D1, NM_179448.3), and *H. sapiens* (NSDHL, NP_057006.1) were cloned from cDNA. Trizol was used to isolate total RNA from the respective species according to the manufacturer's recommendations, which was then followed by cDNA synthesis using SuperScript III Reverse Transcriptase (Thermo Fisher Scientific). All sterol C4-decarboxylase genes were cloned into the Pesc-his vector (Agilent) using NotI and SpeI restriction sites.

Chimeric *SQS* genes were produced using an assembly PCR strategy described by Niehaus et al [33]. For instance, the Algal *SQS* with a yeast C-terminal domain (BS *SQS*) was created by first amplifying the *B. braunii* *SQS* catalytic domain with a 3' extension corresponding to the 5' sequence of the *S. cerevisiae* *SQS* C-terminal domain, and the C-terminal domain of the *S. cerevisiae* *SQS* with a 5' extension corresponding to the 3' end of the algal catalytic domain. The two PCR amplification products were then purified and used in a subsequent PCR reaction with the flanking primers. The gene composed of an algal *SQS* with a yeast hinge motif (BSB *SQS*) was then created in a similar manner using the previous construct as a template.

SQS C-terminal domains used to inhibit yeast growth were amplified from the corresponding full-length *SQS* genes and ligated into the Pesc-ura vector using the pGAL10 promoter for 1x expression. The C-terminal domains were then amplified again and inserted in the reverse orientation relative to the pGAL10 construct, but under the control of the pGAL1 promoter for 2x expression. Chimeric C-terminal domain expression vectors were amplified from the full length chimeric genes.

Yeast Transformation

The recombinant vectors were transformed into each yeast line using a modified version of the lithium acetate method [6]. To produce competent cells, a 2 mL starter culture was inoculated with a single transformed yeast colony, grown for 2-3 days at 28°C shaking at 200 rpm, and used to establish a 50 mL culture of YPDE media. The 50 mL culture was grown overnight in the same conditions to early exponential phase. The cells were briefly centrifuged and resuspended in 25 mL of a 1.2 M sorbitol wash buffer (1.2 M sorbitol in 0.1 M phosphate buffer at pH 7.5). This was followed by a second centrifugation and resuspension in 2.5 mL LioAC-PEG transformation buffer (0.1 M lithium acetate, 1 mg denatured sperm DNA, 34% PEGmW3350). Plasmid DNA (~1 µg) was combined with 200 µL of competent yeast and incubated at 30°C for 1 hr prior to a 10 minute 42°C heat shock. Transformed yeast were plated onto SCE glucose media lacking specific amino acids to select for auxotrophic markers. Yeast lines were confirmed to possess the various expression vectors by colony PCR and stored as glycerol stocks at -80°C.

Spot Plate and Growth Curve Analysis

Yeast growth was analyzed using both spot plating and growth curves. For spot plate analysis, 2 mL starter cultures representing three independent colonies from each transformation were grown to stationary phase in SCE glucose (3 days) and rinsed with sterile water. The cultures were then diluted to an optical density of 1 at 600nm and 5x serial diluted three times using the cuvette station of a NanoDrop 2000c UV-Vis Spectrophotometer (Thermo Scientific) to produced four different optical densities: 1, 0.2, 0.04, and 0.008 at 600 nm. Four to five µL of each dilution were spotted on solid SC glucose or SC galactose media without specific amino acids for selection, with or without exogenous sterol. Photographs were taken after 5-7 days of incubation at 28°C (Fig. 2.1).

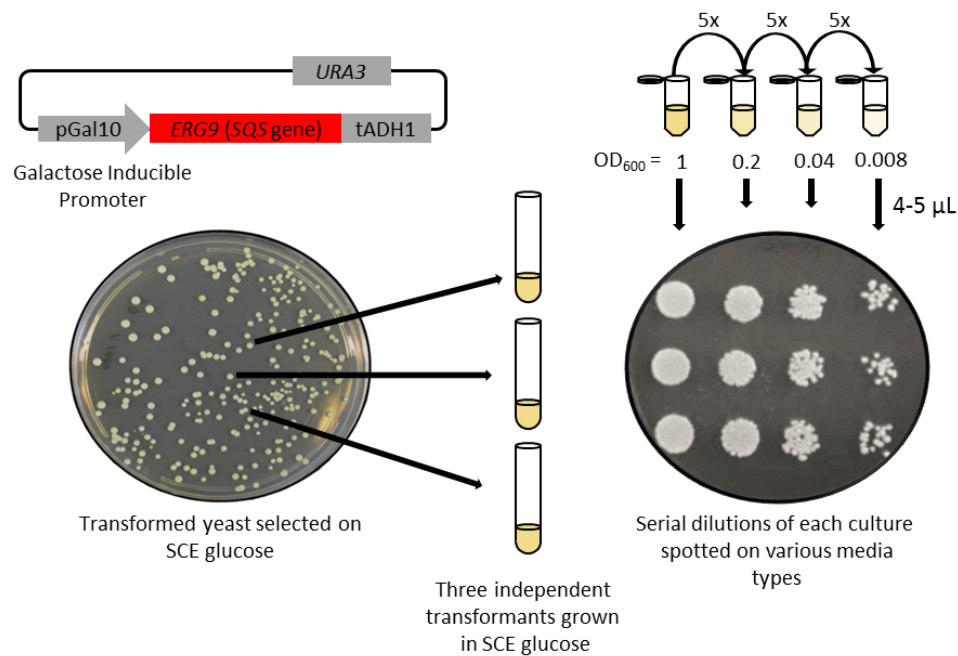


Figure 2.1: Protocol for yeast spot plate analysis. Three 2 mL starter cultures representing independent colonies from each transformation were grown to stationary phase in SCE glucose and rinsed with sterile water. The cultures were then diluted to an optical density of 1 at 600nm and 5x serial diluted three times to produced four different optical densities: 1, 0.2, 0.04, and 0.008. 4-5 μ L of each dilution were spotted on various media types and photographs were taken after 5-7 days of incubation at 28°C.

For growth curve analysis, single colonies from a yeast transformation were used to inoculate starter cultures which were grown in 14 mL culture tubes at 28°C shaking at 200 rpm. Once the starter cultures reached stationary phase (3 days), they were used to inoculate 25 mL of liquid selection media containing glucose or galactose as the sole carbon source. Cultures were grown at 28°C shaking at 200 rpm, and the optical densities were recorded at each time point.

Sterol Profile Analysis using GC-MS

For saponified sterol profiles, yeast were extracted based on a modified method by Quail and Kelly [34]. Cultures were started with single colonies grown in 2 mL of SCE glucose media to stationary phase, and these starter cultures were then used to inoculate 50 mL of SCE galactose induction media. Once yeast reached roughly mid-exponential phase ($OD_{600} = 5-8$), a 20 mL sample was collected at a normalized optical density equal to 5 at 600 nm. Whole cells were resuspended in a saponification solution (2 mL of 60% KOH, 1 mL of 1% pyrogallol, and 2 mL of methanol) in 24 mL glass vials with a screw cap, heated in an 80°C water bath for 2 hours and extracted three times with 5 mL heptane.

To identify carboxysterols, yeast were grown and collected as described above. The cultures were then extracted according to the method of Bligh and Dyer [35]. Cells were collected by centrifugation for 10 min at 400xg, rinsed with sterile water, and resuspended in a 24 mL glass vial with 1 mL of methanol and 0.5 mL of chloroform. Four hundred μ L of glass beads were added to the vial, which was vortexed at room temperature for 6 min at high speed. One-half mL of chloroform and one-half mL of ddH₂O were added to the vial followed by vortexing for an additional 1.5 minutes. The vial was centrifuged at 5,000xg for 4 minutes at room temperature to separate the two phases, and the lower chloroform layer was collected with a glass syringe and transferred to a clean vial. Samples from both extraction methods were evaporated to dryness under nitrogen and derivatized using MSTFA + 1% TMCS (Thermo Scientific) in heptane.

GC-MS analysis was performed on an Agilent 7890A gas chromatograph with splitless injection coupled to an Agilent 5970C inert XL mass spectrometer with a triple-axis detector and an Agilent 19091S-433 capillary column (30 m x 250 μ m). The oven was programmed to hold at 70°C for 2 minutes and then ramped to 270°C at a rate of 20°C/min. Helium (10 psi) was used as the carrier gas, the electron ionization energy was 70 eV, and the inlet temperature 250°C.

Identification of sterols was achieved using purified squalene, lanosterol, zymosterol, and ergosterol standards, and the NIST (National Institute of Standards and Technology) reference database.

Protein Sequence Alignments and SQS Modelling

The full-length SQS and C4-decarboxylase protein sequences, as well as the 26 amino acid hinge region of SQS were aligned using Vector NTI Software (Life Technologies). The full-length yeast SQS protein structures from each kingdom of life were modelled with the help of the Zhan laboratory [36]. Homology models were created using the Protein Modeling module of Discovery Studio (version 2.5.5, Accelrys, Inc.) and the available crystal structure of *H. sapiens* SQS [37]. The missing C-terminal residues were modelled using the QUARK ab initio server.

Western Blot Analysis

Yeast line ZXB ($\Delta erg9,1$) was transformed with Pesc-ura vectors containing amino-terminal FLAG-tagged *S. cerevisiae* or *B. braunii* SQS genes. Three independent transformants were selected and inoculated into 3 mL of SCE glucose media. These starter cultures were grown at 28°C shaking at 200 rpm for three days. A 25 mL culture of SCE glucose media was then inoculated 1:10 with 2.5 mL of the starter culture and incubated shaking at 28°C for 24 hours. Five mL of the culture were centrifuged for 10 minutes at 400xg, resuspended in 50 μ L of ddH₂O and combined with 10 μ L of a 6x Laemmli buffer. The sample was boiled in a water bath for 15 minutes and frozen at -80°C. The remaining culture was centrifuged, the cell pellet washed, and the cells were resuspended in SCE galactose media. After 3 days of induction, 5 mL of sample were removed from the culture and prepared in the same manner as the glucose sample. Samples were then thawed on ice and 25 μ L of each sample was loaded into an SDS PAGE gel along with 10 μ L of SeeBlue Plus2 Pre-stained Protein Ladder (Thermo Fisher Scientific). The protein was transferred to a nitrocellulose membrane and visualized using a monoclonal anti-FLAG antibody M2-alkaline phosphatase antibody and an anti- β actin loading control (Abcam).

Activity Assays

The various SQS genes were transformed in the yeast line ZXB ($\Delta erg9,1$) and liquid cultures were grown in SCE galactose induction media for 4 days. Microsomes were prepared

according to the methods of Pompon et al. (77). Enzyme assays contained microsomes (0.2 mg of membrane protein), 50 mM Tris-HCl, pH 7.4, 20 mM MgCl₂, 5 mM NADPH and 30 μM FPP (S.A. ≈ 1250 DPM/pmol) in a total volume of 50 μL. The reaction mixture was incubated at 37°C for 1 hour followed by termination with 50 μL of 0.5 M EDTA. Hydrocarbons were extracted with 200 μL hexane and used for TLC (silica gel 60 plates, hexane solvent). The squalene zone was scraped and quantified by liquid scintillation spectroscopy. Enzyme activity (pmoles/hr/μg total protein) is recorded as a percent of yeast *S. cerevisiae* SQS activity.

Chemical Profiling for Squalene Accumulation

All chemical profiling was performed in the ZXB yeast line. Three biological replicates were selected to inoculate 3 mL starter cultures of SCE glucose media. The cultures were grown for 3 days at 28°C shaking at 220 rpm. Thirty μL of this culture was then used to inoculate 3 mL of SCE galactose media in a 14 mL culture tube, and the culture was grown at room temperature for 7 days shaking at 220 rpm. To extract hydrocarbons, 1 mL of culture was combined with 1 mL of acetone in a 4 mL glass screw cap vial and vortexed periodically for 20 minutes. One mL of hexane was then added and the vial was vortexed periodically for 30 more minutes. After separation of layers, 350 μL of the hexane phase was added to a 2 mL GC vial and evaporated under nitrogen. Fifty μL of iso-octane was then used to reconstitute the sample and transfer it to a 250 μL GC vial insert. One μL was analyzed via GC-MS.

Chapter 3: Phenotypic Outcomes from Expression of Heterologous Squalene Synthases in Yeast

Introduction

A common method used to characterize a gene in any kingdom of life is functional complementation, adding DNA to a model organism that has a loss-of-function phenotype to see if the gene or genes of interest are able to restore the model organism to its wild-type phenotype. The budding yeast, *Saccharomyces cerevisiae*, and the fission yeast, *Schizosaccharomyces pombe*, are often the model organisms used to test for complementation since genes of interest can be knocked out of the genome by homologous recombination [38]. To design a screen capable of characterizing a human or plant gene in yeast, a 'no growth' phenotype is required that responds under clearly defined conditions, such as allowing putative complemented yeast to incubate under selective growth conditions. To study genes for enzymes like SQS in the sterol biosynthetic pathway, researchers created yeast lines able to use sterols added to growth media under aerobic conditions. This then allowed for the addition of knockout mutations [27,39,40], and growth was inhibited when the sterols were absent from the media unless the expressed ortholog complemented the knockout mutation.

The initial discovery and characterization of the genes encoding SQS (*ERG9*) occurred in the yeast *S. cerevisiae*, followed by *S. pombe*. Together, these organisms paved the way for the preparation of *ERG9* knockout yeast lines ($\Delta erg9$) to verify the identity of genes from both plants and animals [27,41,42]. Yet when the *SQS* genes identified in both humans and the model plants *Arabidopsis thaliana* and *Nicotiana benthamiana* were expressed in $\Delta erg9$ yeast lines, they were unable to complement the mutation [43,44]. Conversely, expression of fungal *SQS* genes cloned from the dimorphic yeast, *Yarrowia lipolytica*, and more recently *Ganoderma lucidum* were able to complement the $\Delta erg9$ mutation, a finding that suggested that there is a unique component of a fungal *SQS* that is required for *SQS* to function properly in yeast [45,46].

Robinson *et al.* first investigated whether or not there is a protein motif or domain required for the complementation phenotype [27]. These investigators reported that the human *SQS* protein displayed only modest levels of enzyme activity in yeast and that this activity was insufficient to complement the $\Delta erg9$ mutation. However, when a chimeric *SQS* gene was created producing a protein with the amino-terminal catalytic domain of the human *SQS* protein

linked to the membrane spanning C-terminal domain of the yeast SQS from *S. cerevisiae*, complementation was possible, and enzyme activity in yeast was restored. These authors concluded that the human SQS might be misregulated when expressed in fungi, and the pathway was unable to produce adequate levels of the end product ergosterol.

Years later, Kribii *et al.* showed that SQS from the yeast *S. pombe* and the plant *A. thaliana* are both able to localize to the ER membrane in yeast and produce equivalent amounts of the product squalene [39]. This led to the conclusion that the lack of complementation was not the result of SQS mRNA expression levels or stability, but rather the result of an intrinsic feature of the protein unrelated to its catalytic activity or membrane localization. Using chimeric enzymes in yeast microsomes and the radioactive substrate FPP, it was then demonstrated that yeast expressing a SQS protein with a non-fungal C-terminal domain were unable to convert the squalene into the next two intermediates in the sterol biosynthetic pathway, oxidosqualene and lanosterol (Fig. 3.1). This led to the hypothesis that the C-terminal domain of a fungal SQS is necessary for the enzyme to integrate properly into the sterol biosynthetic complex. In other words, without a component of the fungal C-terminal domain, SQS cannot effectively deliver squalene to the next step of the sterol pathway.

To define the non-catalytic functional role of a fungal SQS in sterol biosynthesis, we evaluated the ability of SQS proteins from three eukaryotic kingdoms of life to complement the $\Delta erg9$ mutation in the yeast *S. cerevisiae*. Using a promoter system capable of both low and high level expression of each SQS gene, we provide evidence that the fungal SQS proteins have a structural feature unrelated to the enzyme's catalytic capacity that is necessary for yeast growth.

Results

Ergosterol prototrophy is restored by low level expression of a fungal or non-fungal SQS

To verify that a non-fungal SQS cannot complement a strain of *S. cerevisiae* with a SQS genetic deficiency, we isolated plant, and animal SQS genes, as well as two recently identified algal SQS genes, and inserted these genes into an expression vector with the galactose-inducible promoter pGAL10. When yeast are grown in media containing glucose, this promoter is greatly repressed, but not completely. Once the yeast are suspended in media containing galactose as the sole carbon source, the promoter is induced and expression levels greatly increase [47].

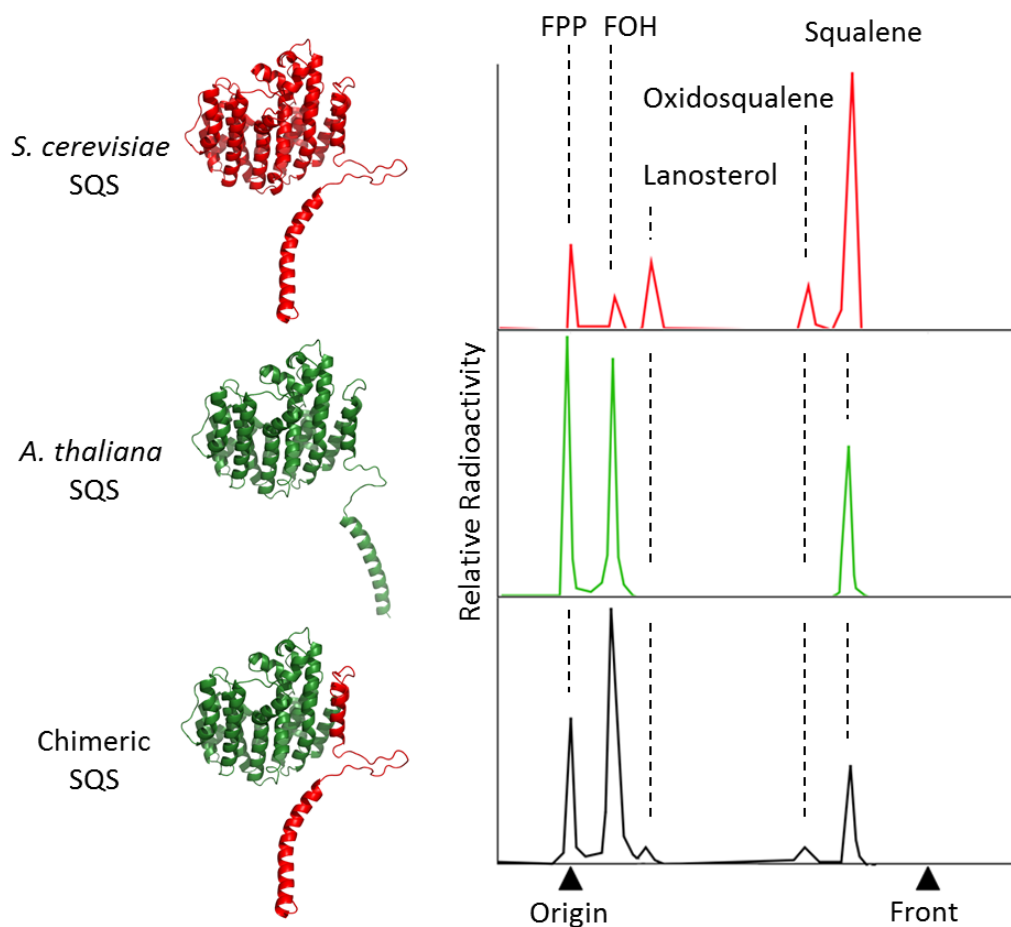


Figure 3.1: The C-terminal domain of a fungal SQS is necessary for accumulation of lanosterol and oxidosqualene. Microsomes were produced from yeast expressing the yeast *S. cerevisiae* SQS (red); plant *A. thaliana* SQS (green); or a chimeric SQS combining the N-terminal catalytic domain of the plant SQS with the C-terminal domain of the yeast SQS. The microsomes were then incubated with the radioactive substrate for SQS, farnesyl diphosphate (FPP), and sterol extracts were analyzed by TLC. FPP was either dephosphorylated to produce farnesol (FOH) or converted to squalene by SQS. Squalene that enters the sterol pathway is converted to oxidosqualene and then lanosterol. Homology models of yeast and plant SQS proteins are based on a published crystal structure of human SQS. This figure is modified from Kribii *et al.* (1997).

Fungal genes were also isolated in order to compare expression of the non-fungal and fungal SQS enzymes. We transformed these constructs, along with an empty expression vector, into the yeast line ZX178-08. This line was produced using EMS mutagenesis to obtain a yeast line capable of aerobic sterol uptake, followed by complete replacement of the native *SQS* gene with a hygromycin selection marker using homologous recombination ($\Delta erg9$) [30]. Transformants were selected for their ability to grow on selective complete (SC) media containing glucose and exogenous ergosterol (SCE glucose), and three colonies from each transformation were selected to be biological replicates. These colonies were grown in liquid SCE glucose media and 5-fold serial dilutions were spot plated on to media containing either glucose or galactose as a carbon source, with or without exogenous ergosterol (Fig. 3.2).

While yeast transformed with each of the *SQS* genes grew robustly on selection media containing glucose and exogenous ergosterol, the observed growth in glucose media without added ergosterol was unexpected. Expression of a fungal *SQS* protein from *S. cerevisiae*, *Candida albicans*, or *Aspergillus nidulans* was able to complement fully the knockout mutation even at these low expression levels. Since it has been reported that a mutated *S. cerevisiae* *SQS* with only 5% of the usual enzyme activity was still able to complement the knockout mutation in yeast [39], it is not surprising that very low expression of a highly active *SQS* in glucose conditions allowed the yeast to grow in the absence of added ergosterol. Yeast cells expressing a non-fungal *SQS* gene from *Arabidopsis thaliana*, *Nicotiana benthamiana*, *Panax ginseng*, *Homo sapiens*, or *Botryococcus braunii* were able to grow under these non-inducing conditions, but the growth was greatly impaired.

Low expression of a non-fungal SQS leads to low ergosterol levels and presqualene alcohol

Since growth of yeast expressing a non-fungal *SQS* was compromised in SC glucose, but was not impaired once exogenous ergosterol was provided, we investigated if a lower level of total ergosterol content was responsible for the growth inhibition. Yeast transformed with each *SQS* gene were grown to mid-exponential phase in glucose media with and without added ergosterol. The cells were then rinsed with sterile water, and 5 α -cholestane was added as an internal standard. Each sample was saponified, extracted with heptane and evaporated under nitrogen prior to derivatization and GC-MS profiling. Figure 3.3 compares yeast expressing a representative fungal (*S. cerevisiae*) and plant (*A. thaliana*) *SQS* in each media type, and figure 3.4 compares a non-fungal *SQS* from each kingdom of life in SC glucose media.

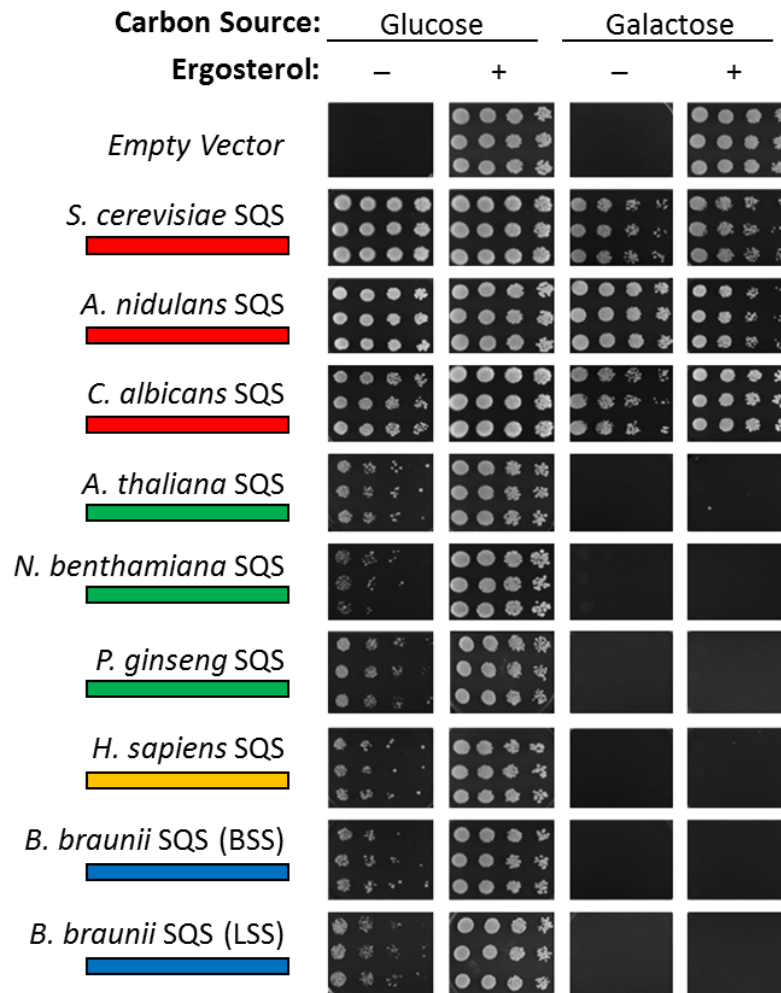
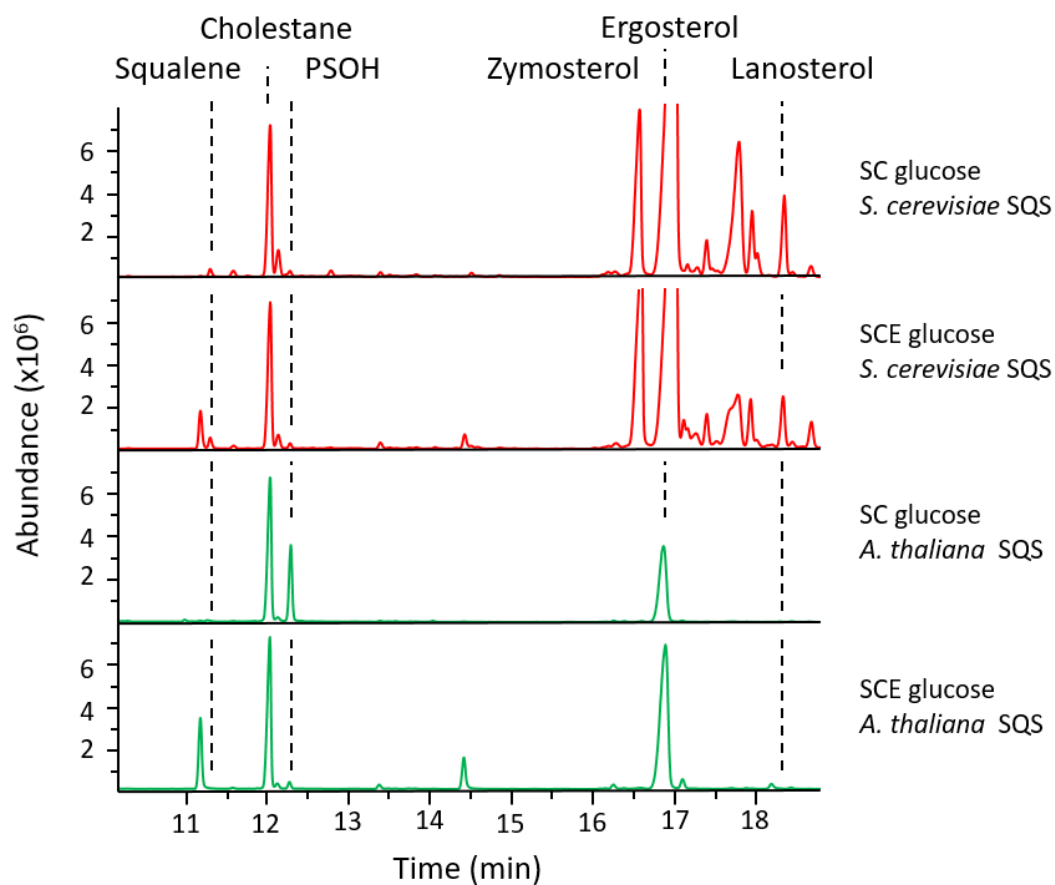


Figure 3.2: Complementation of a SQS knockout ($\Delta erg9$) yeast line with fungal and non-fungal SQS genes is dependent upon expression level. The SQS genes from the fungi, *S. cerevisiae*, *A. nidulans*, and *C. albicans* (red); the plants, *A. thaliana*, *N. benthamiana*, and *P. ginseng* (green); a human (yellow); and an alga, *B. braunii* (blue) from either race B (BSS) or race L (LSS) were cloned under the control of a galactose-inducible promoter and expressed in ZX178-08 yeast ($\Delta erg9$). Three independent transformants for each construct (rows in each panel) were grown in SCE glucose to stationary phase, diluted to a normalized density and 5-fold serial dilutions were plated on media where the promoter is repressed (glucose) or induced (galactose) with or without ergosterol. Images were taken after seven days of growth at 28°C.



Squalene → Lanosterol → Zymosterol → Ergosterol

Figure 3.3: Low-level expression of a non-fungal SQS gene leads to low level accumulation of ergosterol and presqualene alcohol. Sterol profiles were obtained from SQS-deficient yeast expressing the yeast SQS from *S. cerevisiae* (red) or the plant SQS from *A. thaliana* (green) in glucose media with and without exogenous ergosterol. Saponified and derivatized sterol extracts were analyzed by GC-MS. Presqualene diphosphate (PSPP) is an intermediate in the conversion of FPP to squalene and is dephosphorylated in yeast to produce presqualene alcohol (PSOH). 5 α -cholestane was added as an internal standard.

According to the cholestane internal standard, the extraction efficiency was consistently between 89 and 96% for these experiments. SQS-deficient yeast harboring the wild-type SQS gene on a plasmid vector and grown under non-inductive conditions with and without exogenous ergosterol exhibited a sterol profile directly comparable to the sterol profile of the wild-type BY4741 yeast (compare figures 3.4 and 3.5), revealing that a fungal SQS fully recovers sterol pathway functionality. Interestingly, the ergosterol levels were approximately 4 mg/g dry weight whether the *S. cerevisiae* SQS gene was located in its native genomic location or on a plasmid vector. However, higher zymosterol and lower lanosterol accumulation were evident when the gene was expressed from an expression vector.

In conditions where expression of plasmid localized genes would be minimal (SC glucose), yeast harboring the gene for a non-fungal SQS had an ergosterol content that was roughly $1/3^{\text{rd}}$ to $1/5^{\text{th}}$ lower than that of yeast harboring the wild-type *S. cerevisiae* SQS gene. While this showed that the sterol biosynthetic pathway was at least moderately functional, it was interesting to note that no sterol biosynthetic intermediates like lanosterol or zymosterol accumulated above the limits of detection. Instead, a unique peak at approximately 12.2 minutes was observed and identified as presqualene alcohol (PSOH). This identification was based on expression of a previously characterized algal squalene synthase-like enzyme SSL-1 which catalyzes the biosynthesis of presqualene diphosphate (PSPP) that desphosphorylates to presqualene alcohol (PSOH) in yeast due to endogenous phosphatases (Fig. 3.6) [33]. In the SQS catalytic cascade, PSPP is the product of the first half reaction which is not usually released from the enzyme unless the enzyme is NADPH-limited [48]. It is unclear why PSOH might accumulate when a non-fungal SQS is expressed at low levels, but may indicate that there are factors at play that influence the micro-environment surrounding the non-fungal SQS enzymes in the ER membrane. These chemical patterns were unchanged when the yeast were grown in the presence of exogenous ergosterol, except that PSOH did not accumulate as much and the ergosterol content approximately doubled.

These results provide two explanations for why yeast with a non-fungal SQS in SC glucose media might have a decreased growth rate. First, Bell *et al.* reported that the accumulation of PSPP/PSOH can disrupt the normal growth rate of a Δerg9 yeast line [49]. Alternatively, the low level of ergosterol accumulation could be limiting for growth which can be restored by exogenous supplementation of ergosterol but not simple induced expression of the heterologous SQS gene.

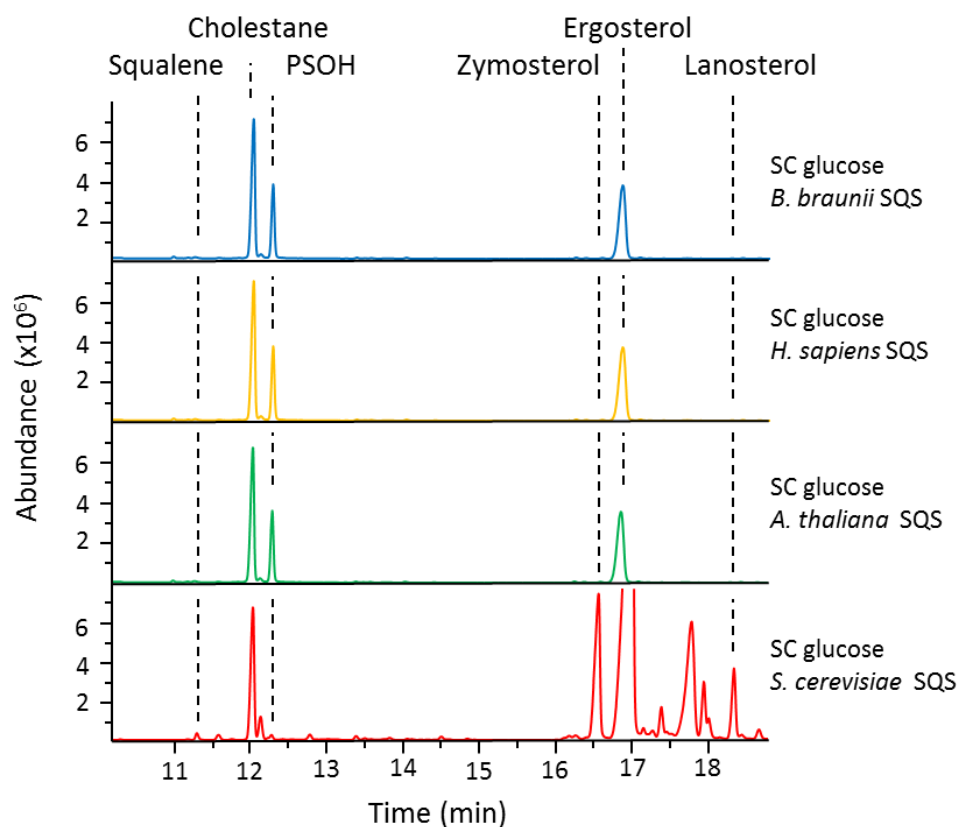


Figure 3.4: The sterol profiles of SQS-deficient yeast expressing non-fungal SQS genes from each kingdom of life reveals the low level accumulation of ergosterol and presqualene alcohol. ZX178-08 yeast expressing SQS genes from each kingdom of life were grown in glucose media with or without exogenous ergosterol. Saponified and derivatized sterol profiles were prepared and extracts were analyzed by GC-MS. The SQS genes were from the alga, *B. braunii* race B (blue); human (yellow); plant, *A. thaliana* (green); and yeast, *S. cerevisiae* (red). 5 α -cholestane was used as an internal standard.

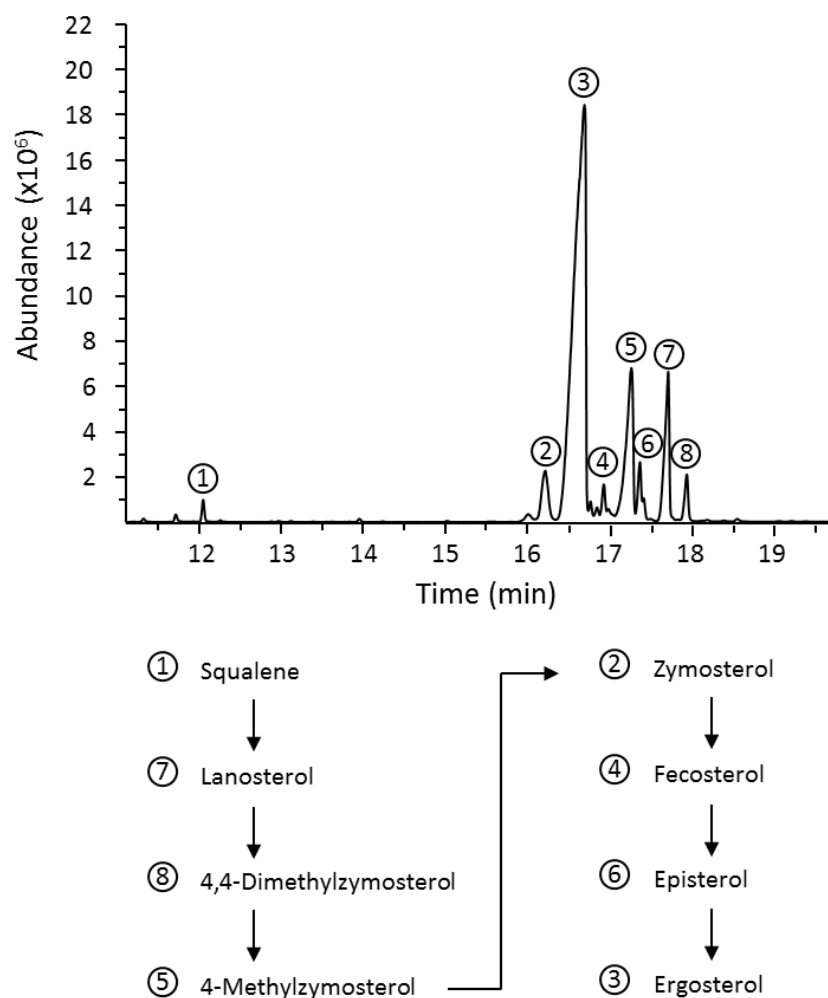


Figure 3.5: Sterol profile of wild-type yeast in SC glucose media. Wild-type haploid BY4741 yeast were transformed with an empty vector and grown on SC glucose selection media without exogenous ergosterol. Yeast were grown to mid-exponential phase; whole cells were saponified; and heptane extracts were derivatized prior to GC-MS analysis. Indicated sterols were identified using purified standards as well as the NIST database.

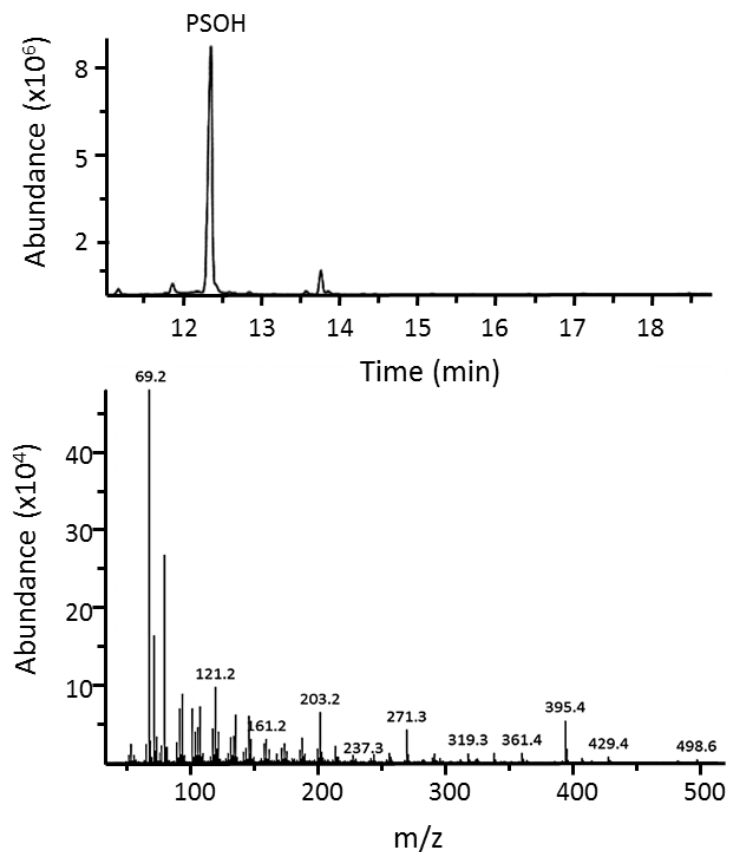


Figure 3.6: Identification of presqualene alcohol in yeast by GC-MS analysis. SQS-deficient yeast were transformed with a fusion enzyme consisting of SSL-1 and SSL-3 from *B. braunii*, as well as an *S. cerevisiae* SQS membrane spanning domain. This chimeric enzyme produces the oil botryococcene, however the SSL-1 enzyme performs the first half-reaction of the enzyme SQS and releases the intermediate presqualene diphosphate (PSPP) which is converted to presqualene alcohol (PSOH) by endogenous phosphatases. Yeast were grown to stationary phase in selection media with added ergosterol, lysed using acetone, and the lysate was extracted with hexane. The extracts were derivatized and analyzed by GC-MS.

Characterizing non-fungal SQS expression, activity, and localization

If low levels of ergosterol are the cause of slow growth, then it would follow that the non-fungal SQS enzymes are either producing less squalene for ergosterol biosynthesis, or the squalene produced cannot be properly converted to the end product in the pathway. If non-fungal SQS enzymes are producing less squalene than the native fungal counterpart, they could be poorly expressed, less active, or mislocalized within the ER membrane milieu in yeast. To test this possibility, we expressed a SQS from each kingdom of life, as well as amino-terminal FLAG-tagged versions of each SQS in the ZXB yeast line. This yeast line contains knockout mutations of SQS and the next enzyme in the sterol pathway, squalene epoxidase (*Δerg9,1*) [30]. This allowed us to investigate not only the expression levels and activity of fungal and non-fungal enzymes following induction but also the total amount of squalene produced *in vivo* over time.

To test the expression levels of non-fungal SQS enzymes compared to a fungal SQS, we first grew three independent transformants containing the FLAG-tagged SQS genes in SCE glucose media to mid-exponential phase. A portion of each culture was collected and stored to determine expression levels when the promoter was repressed. The remainder of each culture was rinsed and resuspended in induction media with added ergosterol (SCE galactose). After 60 hours of induction, cells were collected, resuspended in sterile water with Laemmli buffer [50], and boiled prior to western blot analysis using anti-FLAG and anti- β actin antibodies (Fig. 3.7). There were no significant differences between expression levels of fungal vs non-fungal SQS proteins in yeast, and induction of the promoter with galactose increased the amount of SQS protein approximately nine-fold.

Since SQS protein expression levels in this yeast line were not significantly different whether the protein was of fungal or of non-fungal origin, we next determined whether or not the protein activity varied. Three independent transformants of yeast expressing each SQS were grown in SCE glucose media until the cultures reached stationary phase. Sixty hours after galactose induction, microsomes were prepared, and enzyme activity assays were performed with [3 H] FPP and NADPH. Hydrocarbon reaction products were extracted with hexane and separated *via* thin layer chromatography. The zone containing squalene was scraped and quantified by liquid scintillation spectroscopy (Fig. 3.8). When induced, SQS enzyme activity in yeast containing a non-fungal SQS gene was on average 150% of that observed in yeast expressing a yeast SQS gene.

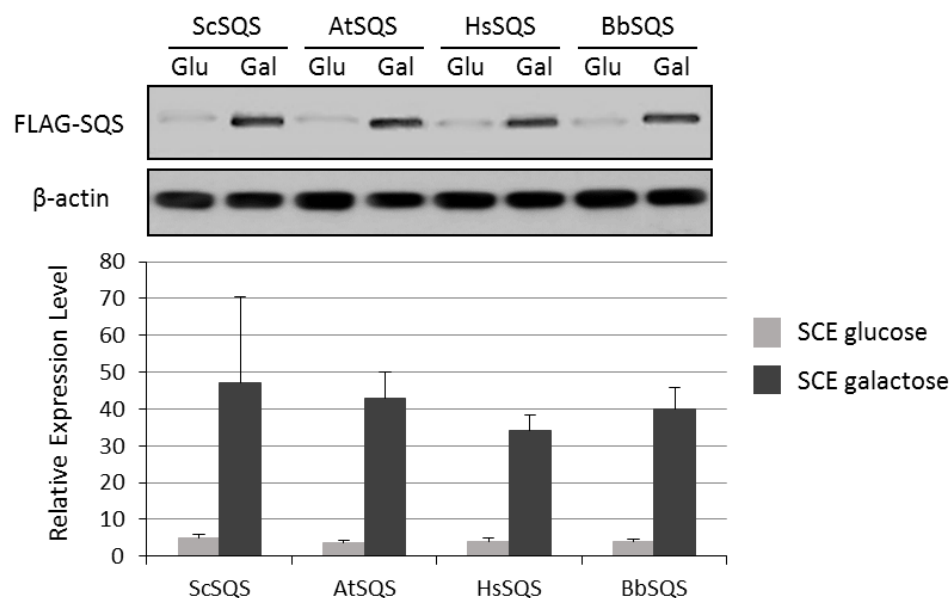


Figure 3.7: Quantification of SQS expression levels from each kingdom of life. SQS-deficient yeast were transformed with N-terminal FLAG-tagged yeast (ScSQS), plant (AtSQS), human (HsSQS) and algal (BbSQS) SQS genes. Three independent transformants for each construct were grown in SCE glucose (Glu) media to repress the pGAL10 promoter, and hence *SQS* gene expression. Aliquots of the cultures collected in exponential phase of growth were switched to induction medium, SCE galactose (Gal), and all the cultures were continued to incubate for 60 hours. Samples were then collected and used for immunodetection of the SQS proteins *via* Western blotting (upper panel). The lower panel represents quantification of the Western blots for all three independent samples.

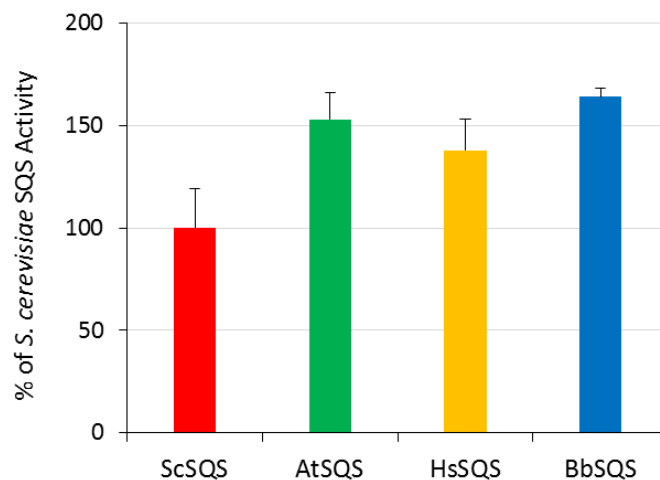


Figure 3.8: Comparison of SQS activity *in vitro* from various kingdoms of life. The yeast line ZXB having both its native SQS gene and squalene epoxidase deleted ($\Delta erg9,1$) was transformed with plasmids harboring SQS genes of yeast (ScSQS, red), plant (AtSQS, green), human (HsSQS, yellow), or algal (BbSQS, blue) origins. Three independent transformants of each were grown in SCE galactose media to mid-exponential phase before microsomes were prepared for *in vitro* assays. Results are presented in comparison (percent) to induced expression of the yeast SQS gene (red).

Squalene levels were also determined by growing each yeast line in SCE glucose or SCE galactose for seven days followed by cell lysis with acetone and hexane extraction (Fig. 3.9). While yeast expressing the *SQS* gene from *S. cerevisiae* produced far higher levels of squalene compared to yeast expressing the non-fungal *SQS* genes in both low and high expression conditions, yeast expressing the *SQS* gene from *C. albicans* produced less squalene than the algal *SQS*. Since both the *S. cerevisiae* and *C. albicans* *SQS* proteins are able to complement the *SQS* genetic deficiency in yeast, the absolute level of squalene production does not appear to be the root cause limiting growth of the yeast transformed with the non-fungal *SQS* genes (Fig. 3.9). While the plant and human *SQS* genes were shown to be expressed at levels comparable to the fungal and algal *SQS* genes following induction (Fig. 3.7), squalene levels were approximately 75% lower (Fig. 3.9). One explanation for this difference may involve the stability of these enzymes in yeast over the course of the seven days that squalene was allowed to accumulate.

These results suggest that all *SQS* enzymes tested are expressed and active *in vivo*. However, expression of a fungal *SQS* appears to favor carbon flux down the sterol biosynthetic pathway (as evident from the lanosterol, zymosterol and ergosterol accumulation patterns in figure 3.3) by a mechanism other than absolute *SQS* enzyme activity.

Overexpression of a non-fungal SQS in Δerg9 yeast prevents growth

As expected, expression of a fungal *SQS* readily complemented the $\Delta erg9$ mutation when the promoter was induced by galactose, and the growth phenotype was largely unaffected by the presence or absence of added ergosterol (Fig. 3.2). However, $\Delta erg9$ yeast expressing a fungal *SQS* gene exhibit some loss of growth vigor (approximately 20%) as noted by a decrease in colony density as a function of serial dilutions. Growth in liquid SCE galactose media revealed that yeast expressing the native yeast *SQS* had a longer lag phase than those harboring the empty vector control, yet the maximum growth rate and the final cell density were directly comparable (Fig. 3.10A).

If a low level of ergosterol accumulation was responsible for the limited growth phenotype observed when the expression vector contained a non-fungal *SQS* gene and expression of the transgene was repressed by growth on glucose, then it would follow that increasing expression of *SQS* by switching the cultures to galactose (inducing) media should improve yeast growth. However, yeast growth was almost completely inhibited once these non-fungal genes were induced for high level expression. Interestingly, this growth inhibition

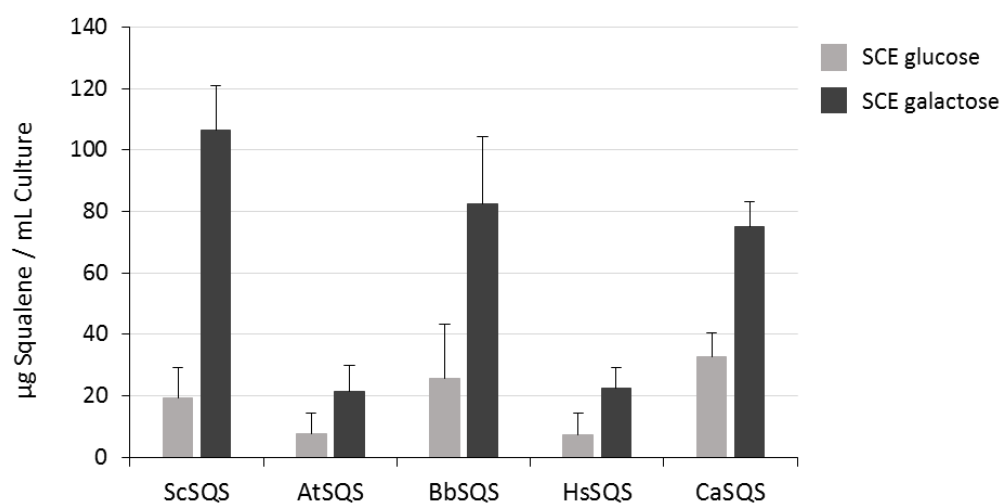


Figure 3.9: *In vivo* squalene accumulation following expression of SQS from various kingdoms of life. The yeast line ZXB having its native SQS and squalene epoxidase genes deleted ($\Delta erg9,1$) was transformed with plasmids harboring SQS genes of yeast (ScSQS or *Candida albicans* CaSQS), plant (AtSQS), human (HsSQS), or algal (BbSQS) origin. Three independent transformants for each were subsequently grown in SCE glucose or galactose for 7 days before quantifying the squalene levels using GC-MS.

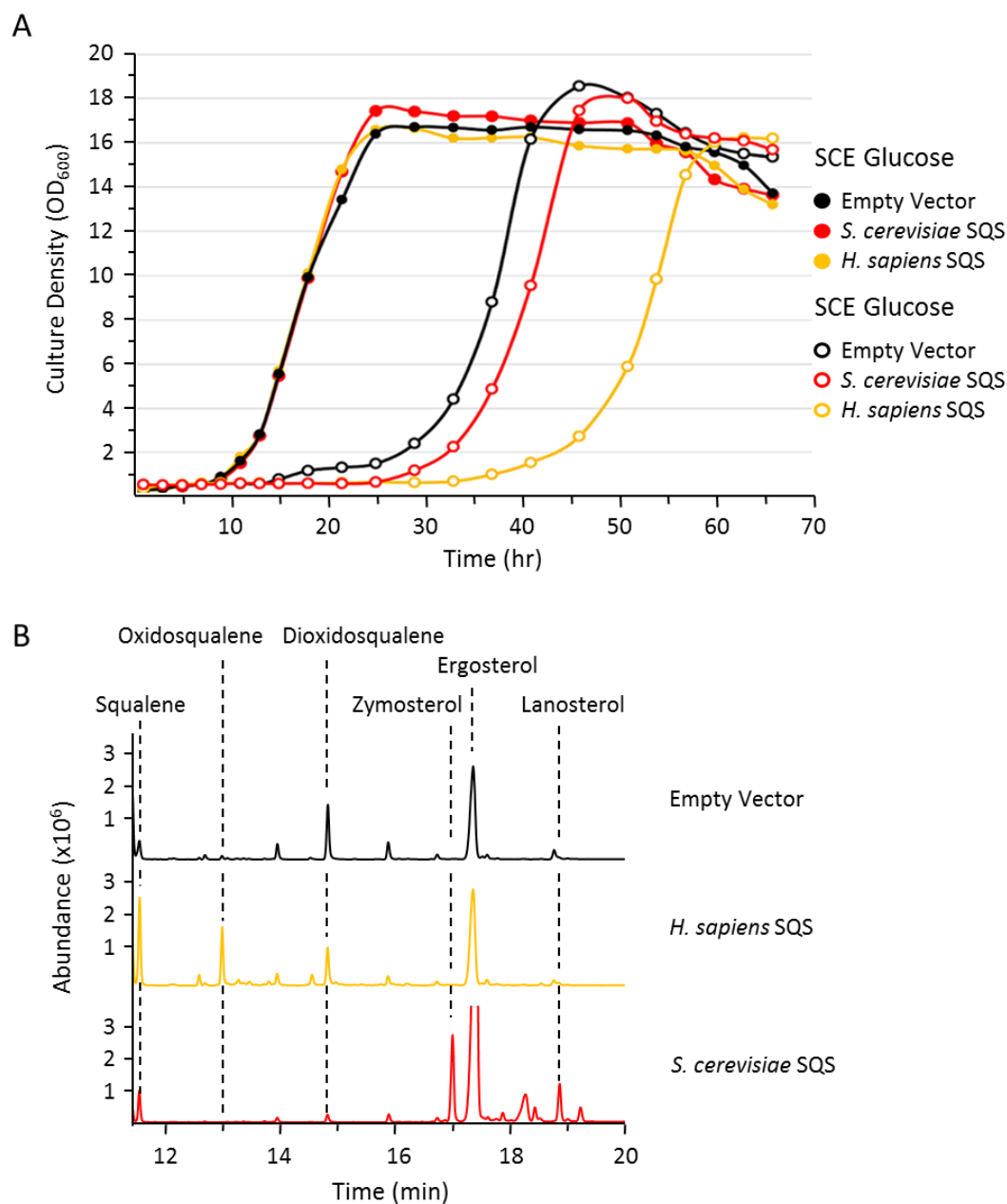


Figure 3.10: Growth and sterol profiles of ZX178-08 yeast cultures expressing wild-type yeast or human *SQS* genes grown in liquid cultures. A) Yeast containing the empty vector or expressing the *S. cerevisiae* *SQS* (red) or human *SQS* (yellow) were grown in glucose (closed circles) or galactose (open circles) media with exogenous ergosterol. The optical density of the culture was recorded every few hours over a period of 65 hours. B) Yeast were grown in SCE galactose to mid-exponential phase, and cells were collected by centrifugation. Non-saponifiable lipids were extracted and derivatized heptane extracts profiled by GC-MS

occurred when expressing the *SQS* genes from *P. ginseng* and *B. braunii* race L, both of which have been shown to complement a *SQS* knockout mutation when induced in liquid media or expressed constitutively. Consequently, there are mutations or growth conditions that do allow for some complementation [51,52].

In these experiments, the only visible yet limited growth occurred on media containing exogenous ergosterol, suggesting that regulation of the sterol pathway or spontaneous mutations may allow the *SQS* genes to complement. Twenty of the colonies able to grow in these conditions were inoculated into liquid media for low level *SQS* expression with exogenous ergosterol (SCE glucose) and then spotted again onto induction media with and without exogenous ergosterol (Fig. 3.11). The non-saponifiable sterols for each of the colonies were also profiled (Fig. 3.12A). All colonies retained the ability to grow on induction media with exogenous ergosterol (SCE galactose) although fifteen of the twenty colonies were unable to grow without exogenous ergosterol (SC galactose). The sterol profiles for 14 of these colonies (represented by Colony 1) were identical to the profiles described previously (Fig. 3.3), while the sterol profile for colony 20 was highly similar to the profile of the *SQS* and lanosterol synthase double knockout yeast line (ZXE, $\Delta erg9,7$) over-expressing an algal *SQS* (Fig. 3.12B). Analysis of the gene for lanosterol synthase (*ERG7*) in this line identified the mutation F445G, and it has been suggested that a mutation from a phenylalanine to a hydrophobic residue at position 445 of lanosterol synthase may disrupt electrostatic interactions in the active site cavity [53]. Four of the twenty colonies (represented by Colony 2) were able to grow in the absence of added ergosterol although growth was impaired. The sterol profiles from each of these lines revealed an accumulation of squalene suggesting a change in squalene epoxidase activity. Sequencing the gene for squalene epoxidase (*ERG1*) in each of these lines revealed the mutations L37P and G247D. Each of these mutations have been shown to decrease enzymatic activity [54,55]

These results were corroborated by the fact that the ZX178-08 yeast line ($\Delta erg9$) expressing an induced non-fungal *SQS* gene is sometimes able to grow in liquid induction media with added ergosterol (Fig. 3.10A); however, analysis of the sterol profiles from these cultures showed only squalene, oxidosqualene, dioxidosqualene, and the ergosterol from the media (Fig. 3.10B). When oxidosqualene accumulates, it is transformed by the enzyme squalene epoxidase to dioxidosqualene, so this profile was likely the result of a change in lanosterol synthase activity. These results argue that over-expression of the heterologous *SQS* enzymes may lead to the build-up of a toxin, and that the toxin may be found in the

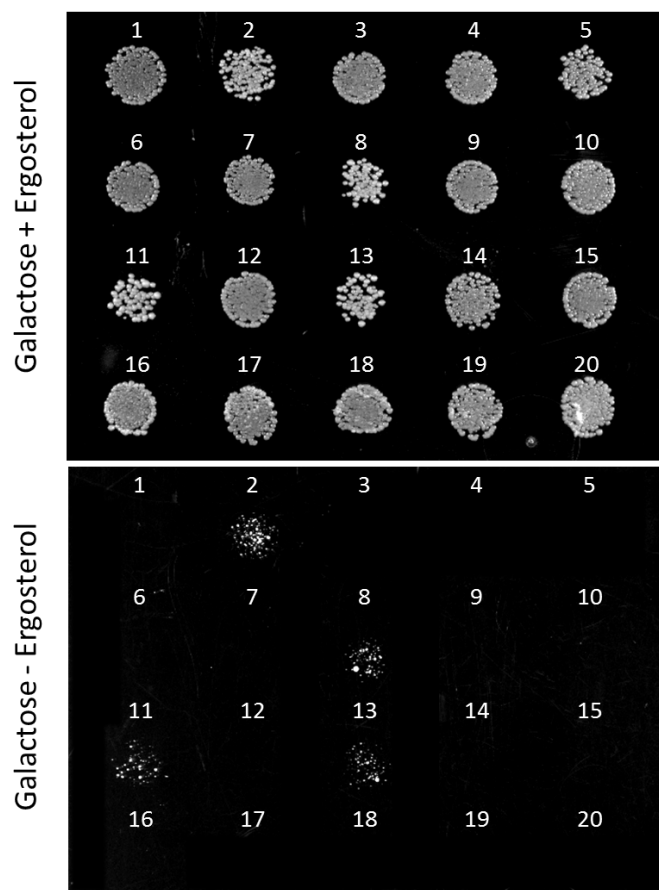


Figure 3.11: Spot plate analysis of twenty colonies expressing an algal SQS with the ability to complement the $\Delta erg9$ mutation in induction media with ergosterol. The yeast line ZX178-08 ($\Delta erg9$) was transformed with an algal SQS gene and plated on media for gene induction (SCE galactose). Twenty colonies were selected, grown in liquid SCE glucose to stationary phase, normalized to optical density and spotted onto induction media with and without added ergosterol. .

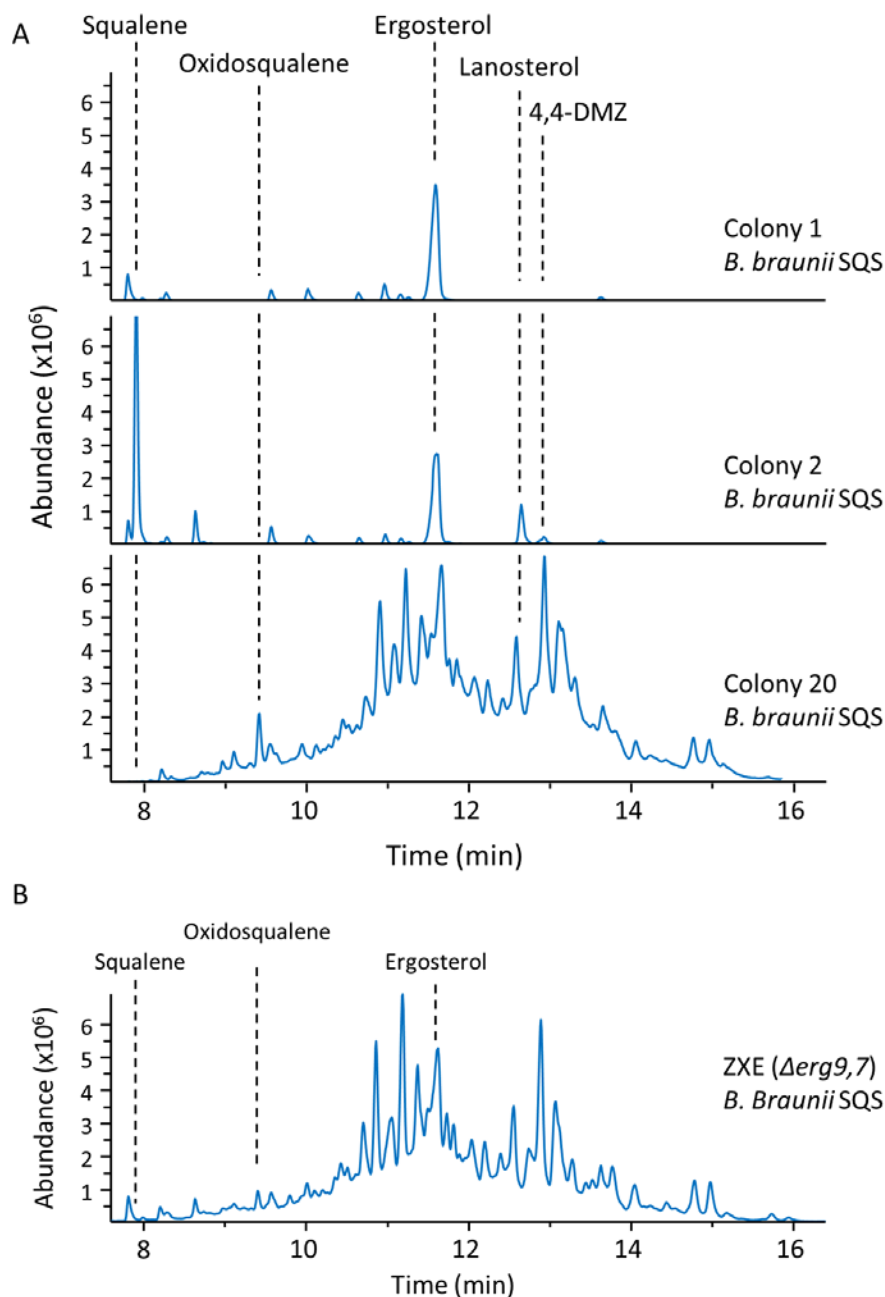


Figure 3.12: Representative sterol profiles for yeast expressing an algal SQS and capable of growing on induction media. A) Yeast from figure 3.11 were grown in liquid induction media with added ergosterol to mid-exponential phase. Cells were collected by centrifugation, saponified and extracted with heptane. Extracts were derivatized and analyzed by GC-MS. B) Yeast line ZXE ($\Delta erg9,7$) was transformed with an algal SQS gene and grown in induction media prior to sterol profiling.

downstream sterol pathway since changes upstream appear to be responsible for the restoration of growth.

SQS biological functions operate via distinct molecular mechanisms

Our results suggest that fungal SQS may possess two functions in the sterol pathway: the first as an essential enzyme for sterol biosynthesis, and the second by providing the means to prevent toxin accumulation. All the non-fungal SQS genes encode for catalytically active enzymes (Fig. 3.8), suggesting that preventing toxin accumulation may occur *via* a molecular mechanism that is not directly associated with catalytic activity. To verify that these functions are independent, we generated point mutations in the yeast and algal SQS genes to disrupt enzyme activity. An inactivating mutation in *B. braunii* race B SQS (A177N) was previously described disrupting the first of the SQS enzyme's two half reactions [49]. By aligning the amino acid sequences of the *B. braunii* and *S. cerevisiae* SQS proteins, the corresponding mutation in *S. cerevisiae* SQS (A183N) was identified and introduced into the yeast gene. In order to verify the effect of these mutations, the genes were co-expressed with the *SSL-1* gene from *B. braunii* race B or an empty vector in the ZXB yeast line ($\Delta erg9,1$). *SSL-1* is able to catalyze only the first half reaction, converting two molecules of FPP to PSPP. Cultures were incubated for seven days; the cells were lysed; and hexane extracts were examined for squalene by GC-MS (Fig. 3.13). As expected, squalene was not detectable when yeast expressed only the inactive yeast or algal SQS enzymes; however, expressing these enzymes with *SSL-1* led to detectable levels of squalene and two additional unknown triterpene peaks (1 and 2).

These inactive SQS proteins were then expressed in combination with active algal and yeast SQS enzymes in the $\Delta erg9$ yeast line. Three independent transformants from each combination were spot plated on media to repress (glucose) and induce (galactose) the promoter, with and without added ergosterol (Fig. 3.14). When expressed singularly, only the fully active, wild-type yeast SQS was able to fully complement the knockout mutation when the yeast were grown in all four conditions, as observed in figure 3.2. The active algal SQS could not fully complement at low expression levels and induction led to complete growth inhibition. Neither the inactive yeast nor the algal SQS enzymes promoted growth of the yeast line, and like the yeast harboring the empty expression vector were only able to grow in the presence of exogenous ergosterol regardless of the induction conditions (glucose vs galactose media).

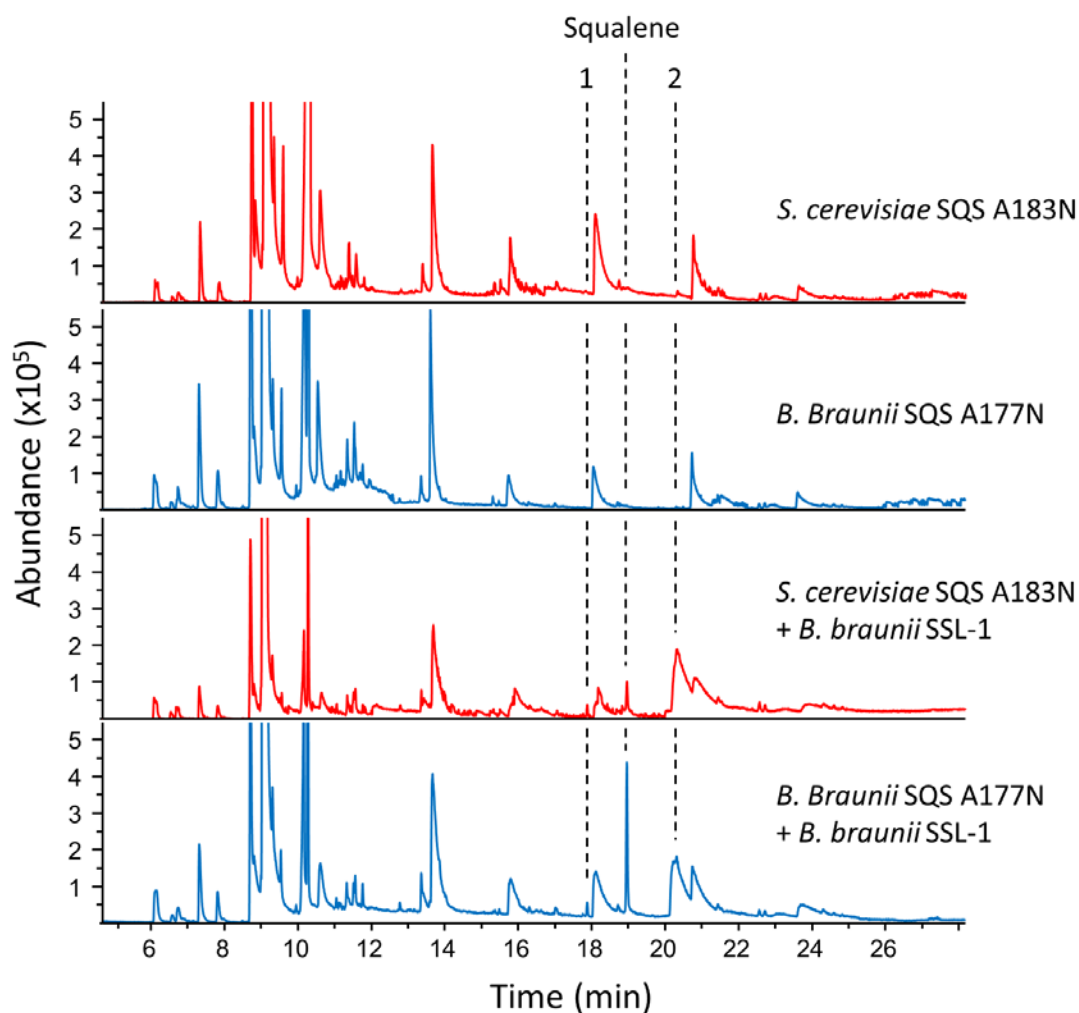


Figure 3.13: Inactive algal and yeast SQS proteins have impaired first half-reactions. Yeast line ZXB ($\Delta erg9,1$) was co-transformed with the the A177N SQS mutant gene of *B. braunii* or the A183N SQS mutant gene from *S. cerevisiae* along with either an empty vector or the *B. braunii* SSL-1 gene encoding a PSPP synthase [33] and either Cultures were incubated at room temperature for seven days, lysed with acetone, and hexane extracts examined for squalene by GC-MS.

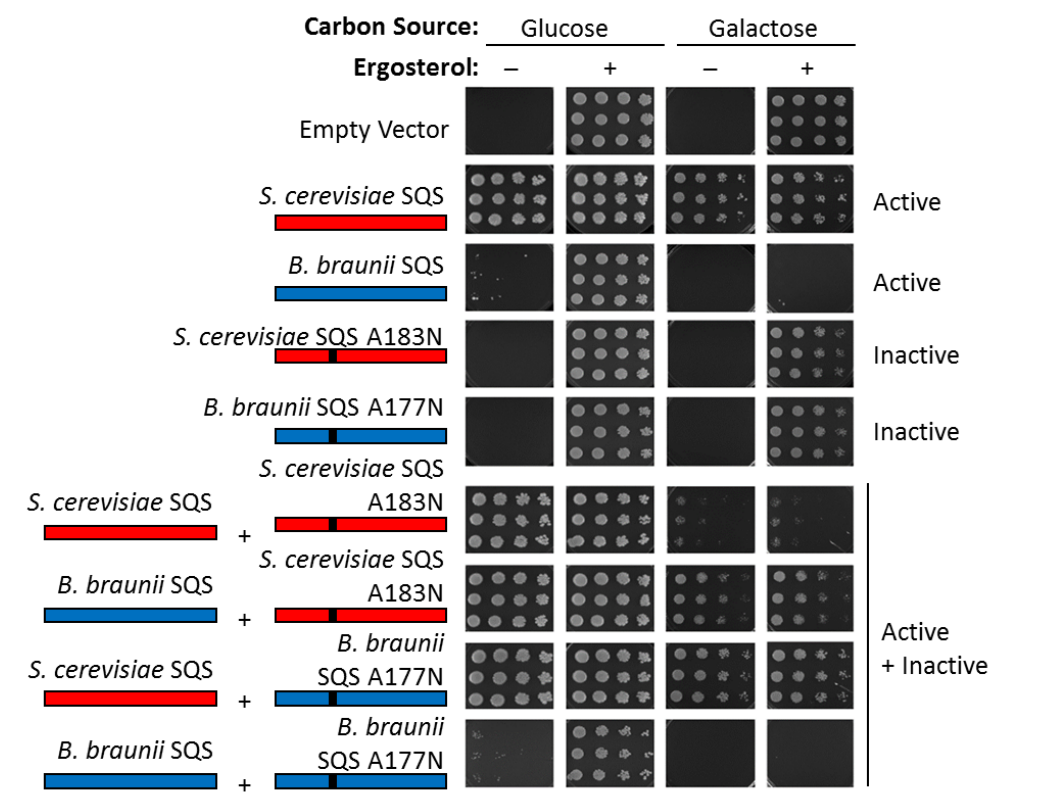


Figure 3.14: Combining an active algal SQS with an inactive yeast SQS recovers the complementation phenotype. Yeast with a $\Delta erg9$ mutation (ZX178-08) were transformed with combinations of active and inactive SQS genes from the yeast, *S. cerevisiae*, and alga, *B. braunii* race B. Independent transformants (rows) were diluted to an optical density of 1 at 600nm. The cultures were serially diluted five-fold (columns) on to the designated media type. Plates were grown for seven days at 28°C before imaging.

As predicted, the combination of an inactive yeast SQS that prevented toxin accumulation with an active algal SQS that produced squalene allowed the yeast to grow as if an active fungal SQS was expressed that was capable of carrying out both functions. This was not the case when co-expressing the algal SQS with an inactive algal SQS. Surprisingly, induction of both active and inactive *S. cerevisiae* SQS genes partially impaired yeast growth. It is possible that very high level expression of a fungal SQS may disrupt interactions in the sterol biosynthetic complex and perhaps even other complexes in the ER membrane. This would delocalize any binding partners and decrease the efficiency of ergosterol biosynthesis. The ability of the A183N mutant fungal SQS gene to support complementation of the $\Delta erg9$ yeast when co-transformed with other non-fungal SQS genes was also extended to the human and plant SQS enzymes (Fig. 3.15).

Sterol pathway analysis identifies a metabolic block after squalene biosynthesis

We next analyzed the sterol biosynthetic pathway in yeast expressing fungal and non-fungal SQS proteins, as well as the combination of an algal SQS with an inactive yeast SQS, following induction. Yeast were transformed with constructs containing galactose-inducible SQS genes from *S. cerevisiae* and *B. braunii* race B, and colonies were used to inoculate flasks of liquid SCE glucose media. Once cultures were grown to mid exponential phase ($OD_{600} = 5$), the cells were collected by centrifugation and rinsed with sterile water before resuspending them in SCE galactose. After inducing for three days, non-saponifiable lipids were extracted with heptane and derivatized samples were analyzed by GC-MS (Fig. 3.16).

Yeast expressing the active *S. cerevisiae* SQS produced ergosterol and many other identifiable sterol intermediates (Fig. 3.16). In contrast, yeast expressing an algal SQS alone produced a low level of both squalene and ergosterol, and no other sterol intermediates accumulated above the level of detection. These results mirrored those seen when expressing each SQS at low levels in glucose conditions (Fig. 3.3), and agreed with the previous findings of Kribii *et al.* that the squalene produced by a non-fungal SQS could not efficiently enter the sterol biosynthetic pathway. It is possible that very low levels of a downstream sterol intermediate are sufficient to impair yeast growth and so this sterol is not identifiable by these methods of analysis. As predicted, the sterol profile of yeast co-expressing the algal and inactive yeast SQS sufficient to impair yeast growth and so this sterol is not identifiable by these methods of analysis. As predicted, the sterol profile of yeast co-expressing the algal and inactive yeast SQS

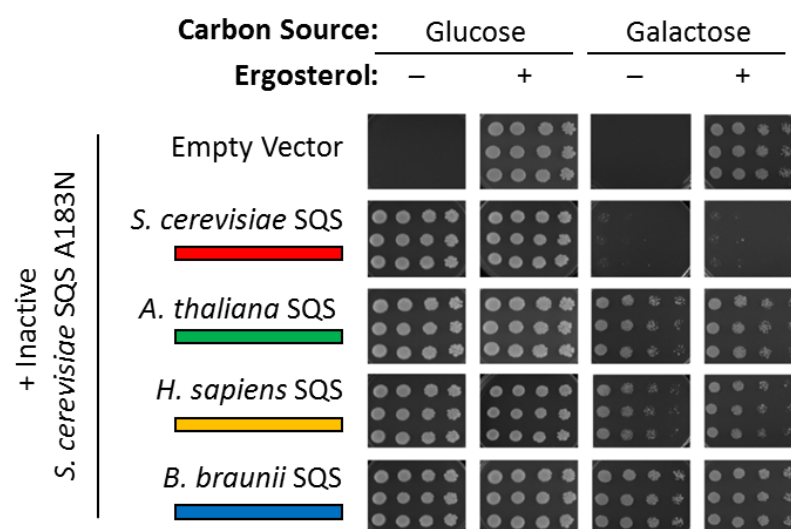


Figure 3.15: Expression of an inactive yeast SQS partially inhibits growth of a $\Delta erg9$ yeast line co-expressing an active yeast SQS while allowing a non-fungal SQS to complement the knockout mutation. Yeast with a $\Delta erg9$ mutation were transformed with an inactive yeast SQS (ScSS A183N) and either a SQS gene from each kingdom of life or an empty vector with a galactose-inducible promoter. Three independent transformants (rows) were grown in SCE glucose liquid cultures for three days prior to normalizing culture density, then spotting five-fold serial dilutions (columns) onto the designated media types. Plates were grown for seven days at 28°C before imaging.

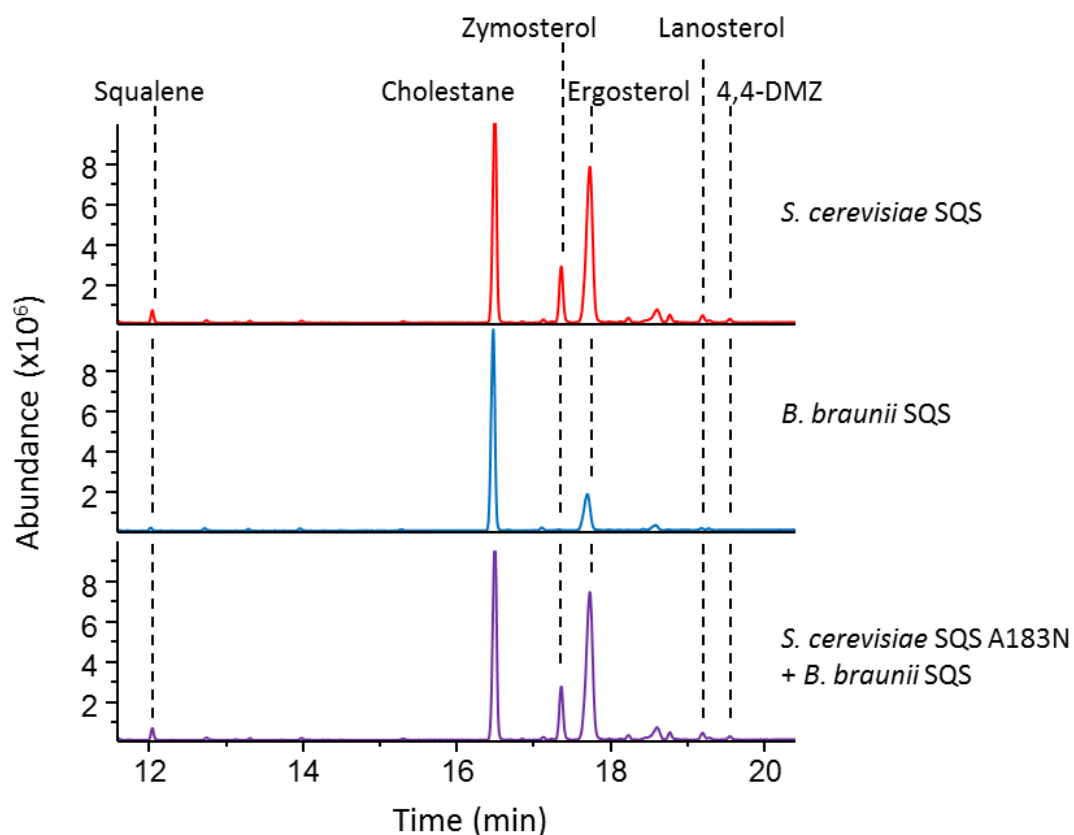


Figure 3.16: The sterol profile of ZX178-08 yeast co-expressing algal and inactive yeast SQS. Yeast line ZX178-08 was transformed with SQS genes from yeast, *S. cerevisiae* (red); alga, *B. braunii* (blue); or a combination of the algal SQS with an inactive A183N mutant yeast SQS (purple) under the control of a galactose-inducible promoter. Yeast were grown in SCE glucose to mid-exponential phase and resuspended in SCE galactose induction media. Cultures were normalized to optical density and nonsaponifiable sterols were derivatized for GC-MS analysis. 4,4-DMZ = 4,4-dimethylzymosterol.

genes mimicked that which was observed when expressing the active yeast SQS alone, demonstrating that the two functions of a fungal SQS can be expressed separately in yeast and fully rescue the sterol biosynthetic pathway.

Discussion

Previous studies have noted that while a non-fungal SQS cannot complement a $\Delta erg9$ mutation, constitutive expression in a double knockout yeast line ($\Delta erg9,1$) allowed significant levels of squalene to accumulate [30]. The results described here extend this observation in distinct ways. Firstly, expression of a non-fungal SQS can partially restore sterol prototrophy and growth to a *SQS* deletion mutant if the heterologous *SQS* gene is expressed at a very low level. While several groups have attempted complementation by expressing *SQS* genes from plants and humans using the strong galactose-inducible promoters, low or leaky expression of these genes under non-inductive conditions (glucose media) without added ergosterol was not tested previously [27,39,40]. This variability in growth does not appear to be caused by a difference in protein expression or activity as shown by both *in vitro* activity assays (Fig. 3.8) and by *in vivo* squalene accumulation (Fig. 3.9).

Initially, we suggested that the impaired growth phenotype in non-inductive conditions could be the result of PSPP/PSOH accumulation or the result of low ergosterol production; however, PSOH was never identified when non-fungal protein expression was induced, suggesting that this intermediate only accumulates when SQS protein levels are low. Similarly, the growth of yeast over-expressing a non-fungal SQS cannot be rescued by adding exogenous ergosterol. We have demonstrated that the non-fungal protein itself is not toxic to yeast growth, as over-expression of an inactive algal SQS does not cause growth inhibition, and yeast lines with a decrease in lanosterol synthase activity are able to grow despite increased expression of an active human or algal SQS (Fig. 3.10 and 3.11).

We suggest that there is a growth inhibitory intermediate or side-product in the sterol pathway that accumulates when a non-fungal SQS is expressed. When low levels of squalene enter the pathway, this intermediate does not accumulate to levels that fully inhibit yeast growth and the ergosterol added to the media is able to dilute the effect of the aberrant sterol in the cell membrane. Increasing expression of the non-fungal SQS, and therefore the level of squalene entering the sterol pathway, leads to such a high accumulation of the toxic intermediate that added ergosterol is no longer able to recover growth.

Chapter 4: Identification of a Metabolic Block Associated with the Heterologous Expression of Non-fungal Squalene Synthases in Yeast

Introduction

By inducing expression of heterologous SQS proteins in yeast with a knockout mutation in the resident *SQS* gene, we observed that expression of a non-fungal SQS leads to cessation of yeast growth even when a suitable source of exogenous sterol is provided. Further analysis suggested that the growth inhibition phenotype could be suppressed by limiting the production of cyclized sterol intermediates. Hence, we sought to identify whether a metabolic block in the sterol biosynthetic pathway caused by over-expression of heterologous *SQS* genes in yeast led to the accumulation of a toxic sterol(s).

Squalene is converted to the end-product ergosterol in yeast through an additional 12 enzymes that are responsible for 15 enzymatic steps (Fig. 4.1 and 4.2). These steps can be divided into two distinct parts [29,56]. The first two steps after squalene biosynthesis form the first cyclized sterol intermediate. Squalene is activated by squalene epoxidase (Erg1) that adds oxygen to the double bond between the second and third carbons (Fig. 4.3) [57–59]. Oxidosqualene is then folded into the active site of oxidosqualene cyclase, also known as lanosterol synthase (Erg7), that catalyzes a complex carbocation mediated cyclization reaction yielding lanosterol. Lanosterol is the first tetracyclic sterol intermediate and the precursor to the diverse array of sterols found in fungi, animals and plants [60,61].

The second portion of the sterol pathway is a “tailoring” section that involves removal of methyl substituents, reducing and introducing double bonds, and addition of methyl and ethyl substituents to create the various sterols found in each kingdom of life. In yeast, this section begins with lanosterol C14-demethylase (Erg11). Removal of the methyl group at the C14 position creates a double bond that is reduced by the sterol C14-reductase (Erg24) to yield 4,4-dimethylzymosterol. The next steps involve three enzymes that make up a C4 demethylation complex. This complex, which has been identified in all kingdoms of life, involves three enzymes held together by the scaffold protein Erg28 [62–64]. As shown in figure 4.4, a sterol C4-methyloxidase (SMO) converts one of the methyl groups in 4,4-dimethylzymosterol to a carboxylic acid intermediate. A sterol C4-decarboxylase (SDC), also known as 3 β -hydroxysteroid dehydrogenase/C4-decarboxylase or NSDHL in humans, affects the oxidation state at the C3

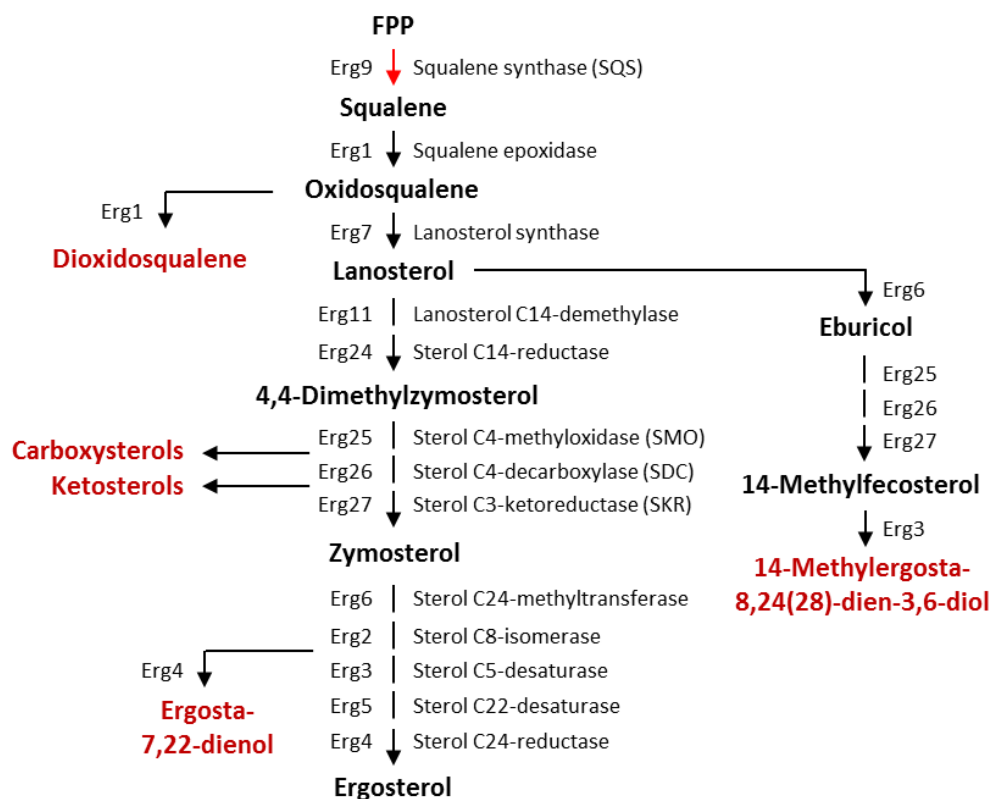


Figure 4.1: Toxic intermediates and side-products of the ergosterol biosynthetic pathway.

Inhibition of various enzymes throughout the fungal sterol pathway are known to cause accumulation of intermediates and side-products (dark red) that inhibit yeast cell growth. 14-Methylergosta-8,24(28)-dien-3,6-diol [65,66], carboxy- and keto-sterols [64,67,68] and ergosta-7,22-dienol [69] have all been associated with toxicity effects in yeast.

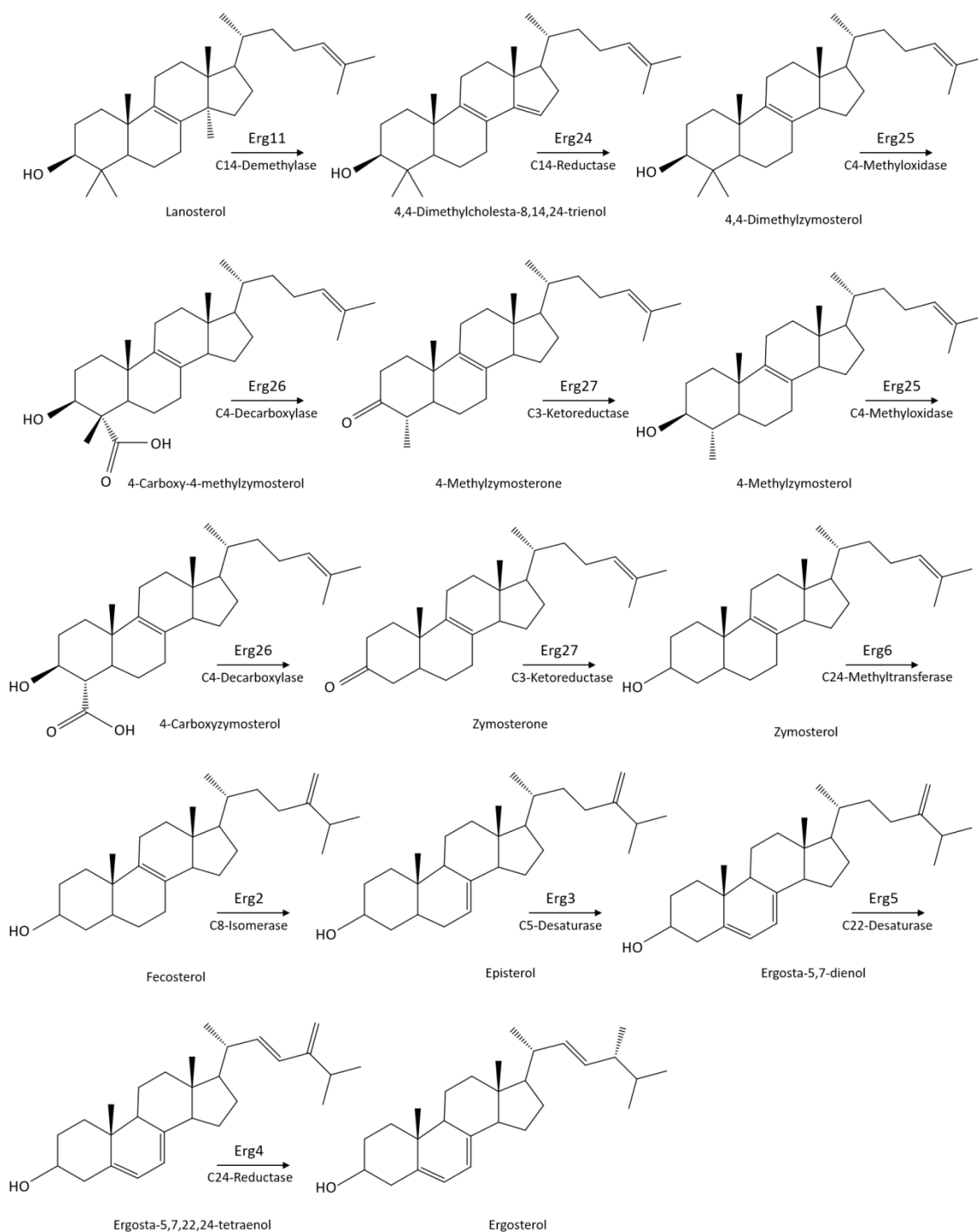


Figure 4.2: The structures of intermediates in the downstream sterol biosynthetic pathway. In fungi, lanosterol is converted to the end product ergosterol through thirteen enzymatic steps performed by ten enzymes.

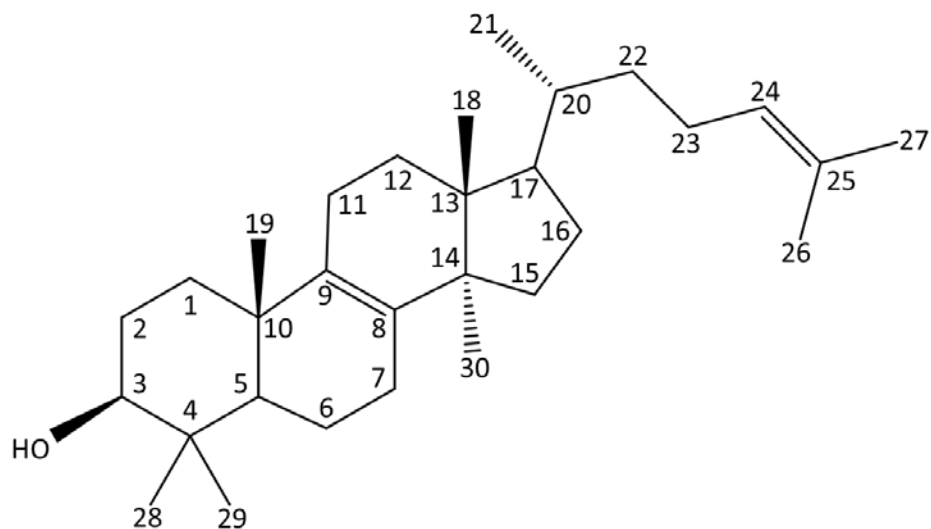


Figure 4.3: The structure, stereochemistry and numbering of lanosterol. Sterols are named according to modifications of the backbone structure according to systematic rules agreed upon by the International Union of Pure and Applied Chemistry (IUPAC). The substituent groups or other structural modifications are indicated by prefixes which include numbers indicative of the position in the carbon skeleton at which the modification occurs.

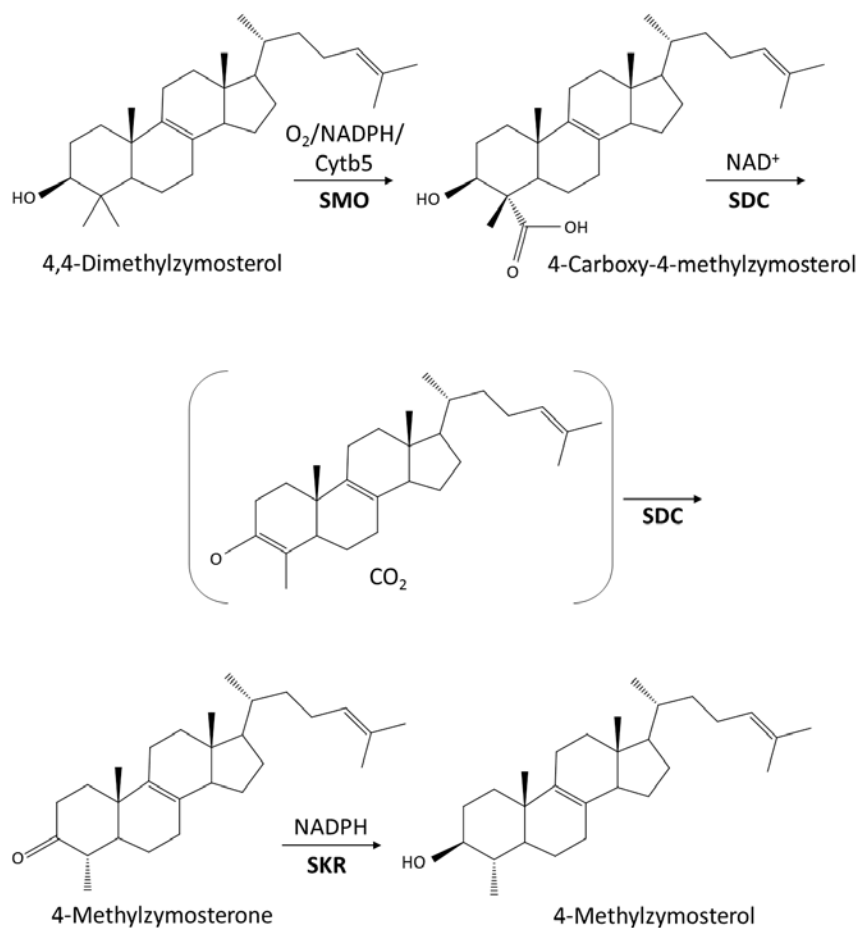


Figure 4.4: Three enzymatic steps in the C4 demethylation complex. The C4 demethylation complex in fungi and human cells consists of three steps that convert 4,4-dimethylzymosterol into a carboxysterol and ketosterol, producing 4-methylzymosterol. The three steps are then repeated to remove the second methyl group at the C4 position.

position resulting in decarboxylation at the C4 position to generate a C3-ketosterol intermediate. The final step is performed by a sterol C3-ketoreductase (SKR) which produces 4-methylzymosterol [29]. This complex of enzymes then repeats this sequence of reactions to remove the second C4-methyl group generating zymosterol. The final five steps of the pathway finish remodeling zymosterol for the production of ergosterol. These steps include a methyltransferase (Erg6), an isomerase (Erg2), two desaturases (Erg3 and Erg5) and a reductase (Erg4).

Due to the importance of sterol metabolism for cell growth and its association with various disorders in humans, systematic mutagenesis and knock-out of genes in yeast have helped to map the biosynthetic pathway and uncovered suspected inhibitory intermediates and side-products. For example, disruption of lanosterol synthase leads to the accumulation of dioxidosqualene which has a regulatory effect in human cells and slows the growth of *S. cerevisiae*. Moving down the pathway, inhibition of the lanosterol C14-demethylase by either a knockout mutation or azole antifungal does not lead to accumulation of its substrate but rather to the development of a complex side-pathway involving sequentially the sterol C24-methyltransferase, the C4 demethylation complex, and the sterol C5-desaturase. This leads to the accumulation of the growth inhibitory side-product, 14-methylergosta-8,24(28)-dien-3,6-diol [65,66]. Inhibition of two steps in the C4 demethylation complex, the C4-decarboxylase and the C3-ketoreductase, lead to intermediates that may disrupt membrane architecture [64,67,68]. The final five steps in the sterol pathway are not necessary for yeast viability; however, knockout mutations involving these enzymes were shown to produce side-products that slow yeast growth. For instance, a mutant involving the sterol C5-desaturase accumulates ergosta-7,22-dienol [70].

In order to identify a region of the sterol biosynthetic pathway that may be responsible for the growth inhibition caused by expression of a non-fungal SQS in *S. cerevisiae*, various SQS genes under the control of a strong inducible promoter, the galactose-inducible gene expression system (pGAL), were analyzed for their impact on the growth of yeast cells in combination with other knockout mutations. By combining this data with additional experiments using antifungal agents and expression of a heterologous squalene epoxidase, a gene that suppressed the growth inhibitory effect of non-fungal SQS expression in yeast was identified and led to the elucidation of the metabolic block and an intermediate that appears to be responsible for a toxic phenotype.

Results

Yeast expressing a non-fungal SQS were rescued by blocking squalene flux

If a toxin is produced in the pathway after squalene biosynthesis, then it follows that blocking squalene flux into the pathway with deletion mutations involving genes downstream of SQS should be able to recover the wild-type growth phenotype in the presence of exogenous ergosterol. For this reason, deletion mutations involving the genes for squalene epoxidase (*ERG1*), lanosterol synthase (*ERG7*), sterol C24-methyltransferase (*ERG6*), and sterol C5-desaturase (*ERG3*) were independently introduced into the SQS knockout yeast line ZX178-08. This created the double mutant lines $\Delta erg9,1$ (ZXB), $\Delta erg9,7$ (ZXE), $\Delta erg9,6$, and $\Delta erg9,3$, respectively. Insertional inactivation of these genes was performed by introducing a G418 resistance cassette surrounded by 5' and 3' segments of the target genes to promote homologous recombination. Each yeast line was then transformed with an empty expression vector or galactose-inducible SQS gene. Three independent transformants for each SQS gene were subsequently plated onto induction media containing ergosterol (Fig. 4.5). The transformed yeast lines $\Delta erg9,6$ and $\Delta erg9,3$ were also grown in liquid glucose media with added ergosterol prior to inducing high level expression of introduced SQS genes. The cells were collected by centrifugation, saponified and the total extractable lipids derivatized for GC-MS analysis (Fig. 4.6).

As expected, all of the yeast lines transformed with the empty expression vector grew well in induction conditions because exogenous ergosterol was included in the media, as did the yeast lines transformed with the *S. cerevisiae* SQS gene. The $\Delta erg9,6$ yeast line expressing a yeast SQS predictably accumulated the intermediate, zymosterol, the substrate for sterol C24-methyltransferase which did not inhibit growth. The growth rate of the $\Delta erg9,3$ yeast line expressing yeast SQS was decreased compared to the other lines. This may be due to the accumulation of ergosta-7,22-dienol. Studies in the yeast *C. albicans* have shown that deletion of the sterol C5-desaturase ($\Delta erg3$) led to the accumulation of this side-product as well as a decreased growth rate [69].

When squalene was blocked from entering the sterol biosynthetic pathway due to an additional knockout mutation involving squalene epoxidase (*ERG1*) or lanosterol synthase (*ERG7*), yeast expressing a non-fungal SQS were able to grow under induction conditions. This suggested that the growth inhibition caused by expression of a non-fungal SQS was not caused

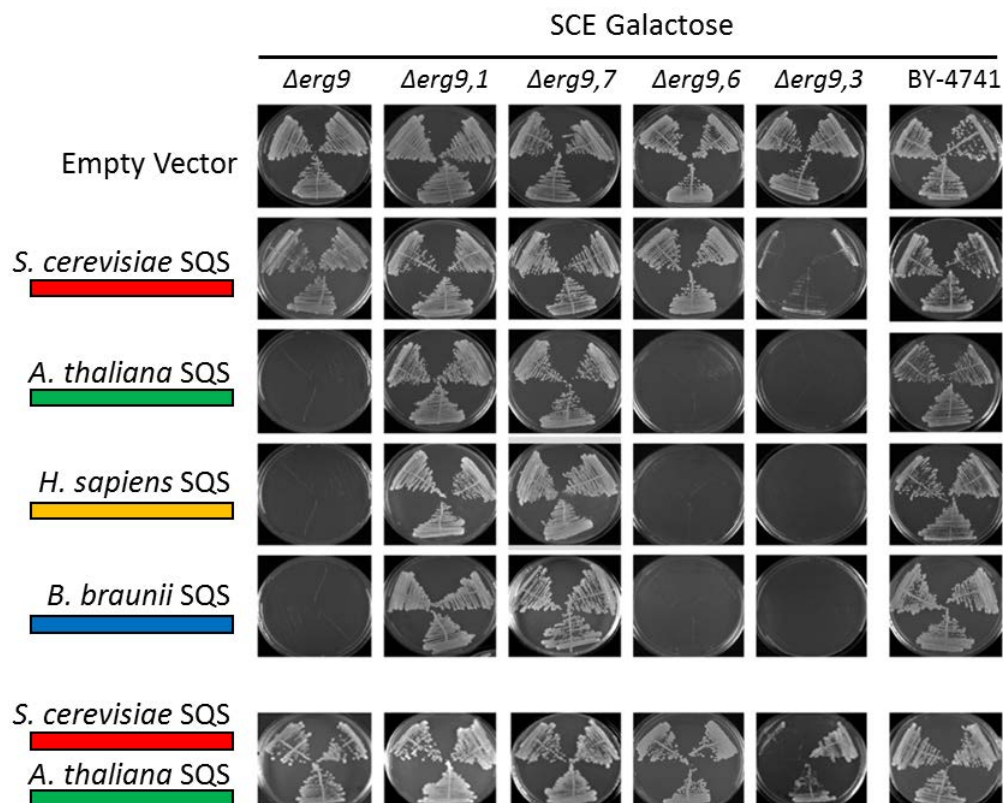


Figure 4.5: Expression of non-fungal SQS, but not yeast SQS, is associated with the production of toxic downstream sterol metabolites. Additional knockout mutations were created in the yeast line ZX178-08 involving the enzymes that appear after SQS in the sterol pathway, squalene epoxidase (Erg1) and lanosterol synthase (Erg7), as well as the sterol C24-methyltransferase (Erg6) and sterol C5-desaturase (Erg3) that appear at the end of the pathway. Yeast lines expressing the indicated SQS genes were grown on non-inducing media (SCE glucose) before three independent transformants for each construct were plated on inductive media with exogenous ergosterol (SCE galactose) and incubated for an additional seven days.

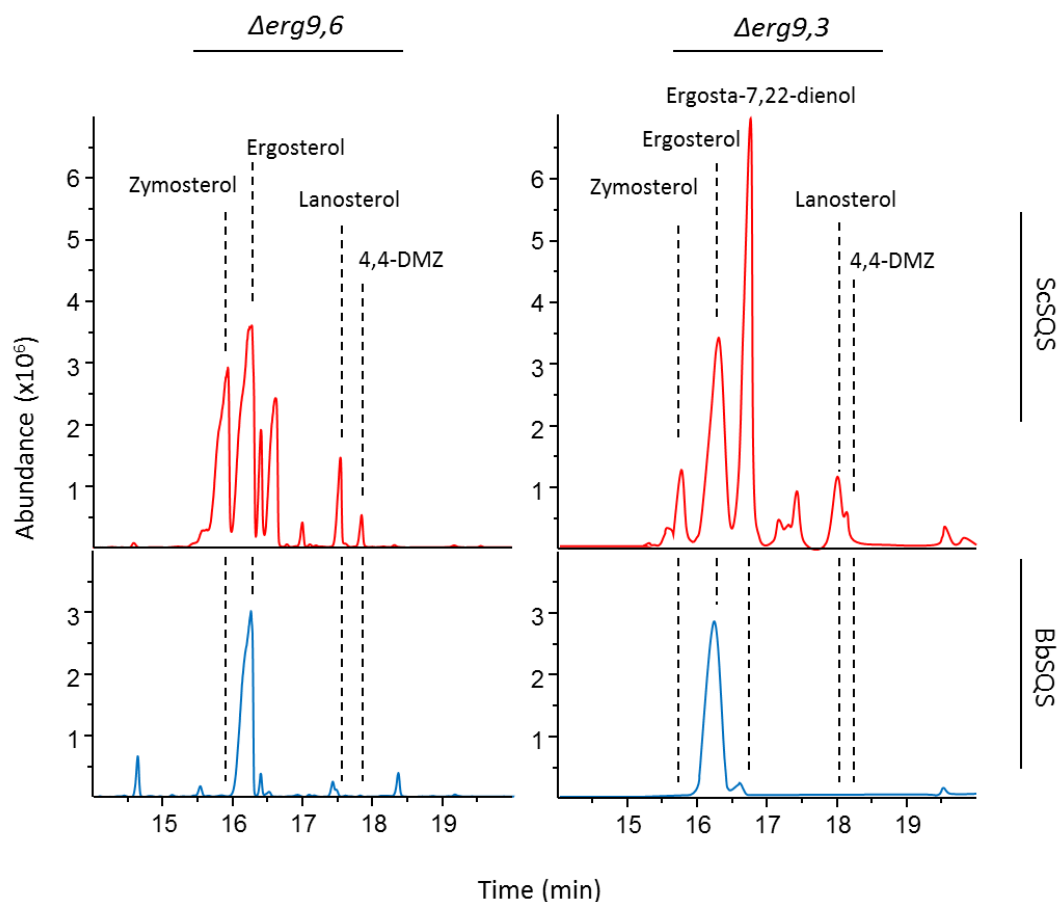


Figure 4.6: Sterol profiles from yeast lines $\Delta erg9,6$ and $\Delta erg9,3$ expressing fungal or non-fungal SQS. Yeast lines $\Delta erg9,6$, and $\Delta erg9,3$ were transformed with either a fungal SQS from *S. cerevisiae* (ScSQS, red) or an algal SQS from *B. braunii* race B (BbSQS, blue). Yeast were grown in non-inductive media to mid-exponential phase before collecting the cells by centrifugation and resuspending them in induction media. The cultures were induced for an additional 30 hours prior to profiling total extractable lipids by GC-MS analysis. 4,4-DMZ = 4,4-Dimethylzymosterol

by accumulation of squalene or oxidosqualene forms but rather by a toxic intermediate or side-product synthesized downstream of lanosterol synthase. Conversely, growth was not recovered by deletion of the sterol C24-methyltransferase (Erg6) responsible for converting zymosterol into fecosterol or the sterol C5-desaturase (Erg3), that catalyzes the production of ergosta-5,7,24(28)-trienol from episterol (Fig. 4.2). Hence, a toxic intermediate or side-product appears to result from a change in metabolite flux in the center portion of the sterol pathway between lanosterol and zymosterol biosynthesis.

The reason for creating a double knockout mutation involving the gene for the sterol C5-desaturase (*ERG3*) was two-fold. Not only did it verify that a toxin(s) was produced upstream of the C5-desaturase in the sterol biosynthetic pathway but also suggested that the metabolic block caused by expression of a fungal SQS was not at the level of the lanosterol C14-demethylase (Erg11). Hull *et al.* showed that *C. albicans* becomes resistant to azoles by decreasing the activity of the sterol C5-desaturase (Erg3) [66]. This led to an accumulation of 14-methylfecosterol instead of a toxic diol, which the cells incorporate into membranes much like ergosterol (Fig. 4.1). Since deletion of the *ERG3* gene did not recover the wild-type growth phenotype following expression of a non-fungal SQS, growth inhibition in this instance does not appear to occur *via* the same mechanism induced by azole antifungals.

Conversion of lanosterol to ergosterol is dependent on a fungal SQS C-terminal domain

While it may seem reasonable that accumulation of a toxic downstream sterol occurs because the non-fungal SQS enzymes generate too much squalene for the downstream sterol pathway compared to fungal enzymes, the results described in chapter 3 showed that complementation of the $\Delta erg9$ yeast line did not correlate with SQS activity level (Fig. 3.8). However, the *in vitro* activity was determined in yeast microsomes, and *in vivo* squalene accumulation in a $\Delta erg9,1$ yeast line. Neither of these experiments allow for the possibility of feed-back regulation. One hypothesis is that a downstream toxin/metabolite accumulates regardless of the origin of the SQS expressed. This toxin/metabolite could then lead to feed-back regulation of a fungal SQS, which may then contain motifs allowing for post-translational regulation of SQS enzyme activity. This would lead to lower squalene levels and control of toxin accumulation. The non-fungal SQS proteins would not be subject to such a post-translational regulation because they lack the protein motifs mediating this regulatory mechanism. This, toxin accumulation would occur leading to growth inhibition.

If this were so, then simultaneous over-expression of both fungal and non-fungal SQS genes would be expected to exhibit the growth inhibition phenotype. SQS activity would be far too great and the non-fungal SQS would not be regulated. When both the yeast SQS gene from *S. cerevisiae* and the plant SQS gene from *A. thaliana* were co-expressed in each of the knockout yeast lines and three independent transformants of each were grown on induction media with added ergosterol, all yeast lines were able to grow equally as well as those lines only expressing a fungal SQS gene (Fig. 4.5).

This unexpected result necessitated a second confirmation. Hence, we sought to express non-fungal SQS genes in an otherwise wild-type yeast line. We transformed BY4741 (which harbors its own genomic SQS gene) with SQS genes from each different kingdom of life and streaked three independent transformants onto induction media with added ergosterol (Fig. 4.5). Interestingly, this yeast line was able to grow regardless of whether a fungal or non-fungal SQS gene was highly expressed. These observations altogether suggested that it was not regulation of fungal SQS activity per se that prevented toxin accumulation in yeast. Instead, there may be a structural feature(s) unique to the fungal SQS that is required to organize the sterol biosynthetic pathway and allow for the efficient flux of sterol intermediates to the end product ergosterol, thus preventing accumulation of a toxic intermediate or side-product.

If a fungal SQS were necessary for efficient production of ergosterol from lanosterol then it would follow that the $\Delta erg9$ yeast line would only be able to utilize exogenous lanosterol for growth when expressing a fungal SQS. Growth of yeast with an empty vector or a non-fungal SQS should be inhibited since lanosterol would be expected to enter the sterol pathway and be converted into the toxic intermediate rather than ergosterol. The ZXB yeast line ($\Delta erg9,1$) was used so that any squalene produced would not be able to enter the sterol pathway, and yeast growth would be dependent on exogenous lanosterol. ZXB was therefore transformed with both fungal and non-fungal SQS genes, as well as a fungal mutant SQS gene (SQS A183N) that encoded for a catalytically impaired squalene synthase enzyme. Three independent transformants for each construct were then spotted onto glucose or galactose media containing either exogenous ergosterol or lanosterol (Fig. 4.7). Because the ZXB line harbors a deletion of the squalene epoxidase gene ($\Delta erg1$), squalene produced via either the fungal or non-fungal SQS would not be able to enter the sterol biosynthetic pathway and hence, all transformed yeast would be expected to be dependent upon exogenous ergosterol to grow. As shown in figure 4.7

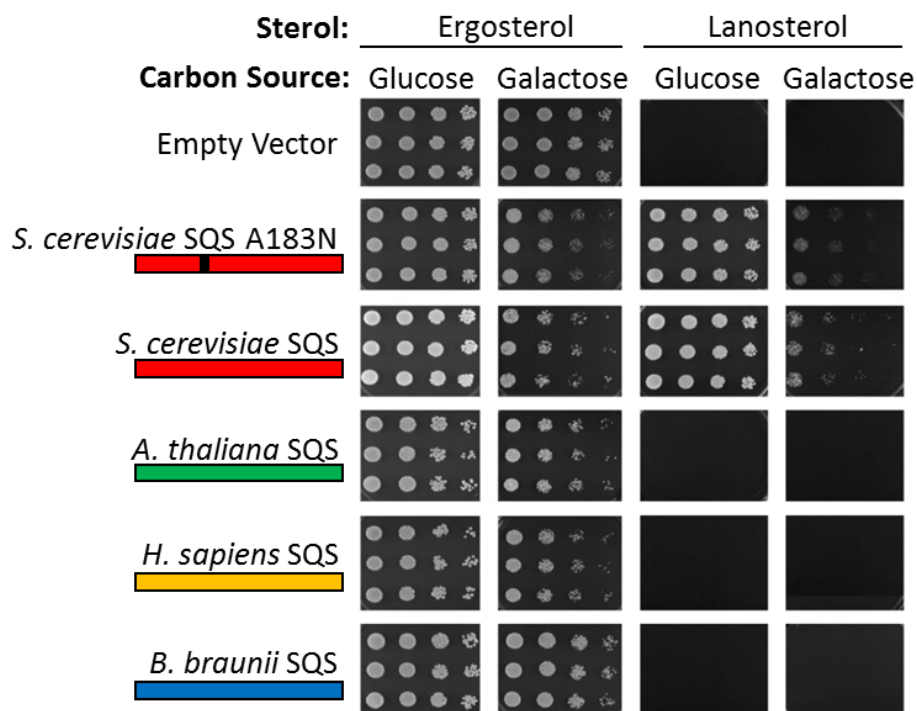


Figure 4.7: Utilization of exogenous lanosterol in yeast is dependent on the presence of a fungal SQS. Yeast line ZXB ($\Delta erg9,1$) was transformed with either an empty vector or a SQS gene from a yeast, *S. cerevisiae* (red); plant, *A. thaliana* (green); human, *H. sapiens* (yellow); or alga, *B. braunii* (blue). An expression vector containing an inactive yeast SQS gene with an A183N point mutation was also transformed. Three independent transformants for each construct (rows in each panel) were grown in SCE glucose to stationary phase, diluted to a normalized density and 5-fold serial dilutions were plated on media where the promoter is repressed (glucose) or induced (galactose) with either ergosterol or lanosterol.

this was the case. When lanosterol was added as the source of exogenous sterol, only yeast expressing an active or an inactive *S. cerevisiae* SQS were able to grow. Growth of yeast expressing either the empty vector or a non-fungal SQS was inhibited.

Manipulating carbon flux identifies a metabolic block in the C4 demethylation complex

In an effort to accumulate enough of a putative toxic intermediate prior to cell growth arrest or feedback inhibition, we used the antifungal agent terbinafine to inhibit the enzyme squalene epoxidase (Erg1). To optimize the concentration of terbinafine used in each culture, $\Delta erg9$ yeast expressing the algal SQS from *B. braunii* race B were grown in induction media with exogenous ergosterol (SCE galactose) and 0-64 $\mu\text{g/mL}$ of terbinafine. The double knockout yeast line ZXB ($\Delta erg9,1$) expressing algal SQS was grown simultaneously without terbinafine as an example of yeast growth when squalene epoxidase activity was absent. Growth was quantified by measuring the optical density at 600 nm over time (Fig. 4.8). While the $\Delta erg9$ yeast cultures without terbinafine did not grow significantly over the course of 45 hours, increasing amounts of terbinafine improved the growth rate. Cultures grown with 64 $\mu\text{g/mL}$ of terbinafine grew at a rate similar to the $\Delta erg9,1$ yeast line, indicating that additional terbinafine would not be able to further over-come the growth inhibition.

Yeast expressing each non-fungal SQS were then grown in SCE galactose media containing 64 $\mu\text{g/mL}$ terbinafine to mid-exponential phase. To remove the terbinafine treatment from the cultures, the cells were collected by centrifugation and resuspended in water six times. The cells were then resuspended in galactose induction media and incubated for various lengths of time before being profiled for their total lipid contents by GC-MS (Fig. 4.9). Prior to rinsing the cells, chromatograms showed that terbinafine was present and squalene greatly accumulated. After washing out the terbinafine, squalene was able to enter the sterol pathway, and cyclized sterol intermediates began to accumulate. For yeast expressing a yeast SQS from *S. cerevisiae*, the sterol profile mirrored that of wild-type yeast BY4741 within three hours. In contrast, only about half of the terbinafine could be removed from the cells expressing a non-fungal SQS suggesting some change in cell permeability. Most striking, only lanosterol and 4,4-dimethylzymosterol began to accumulate by the twelve hour time-point, and no 4-methylzymosterol or zymosterol were detectable.

For the same reason, a non-fungal squalene epoxidase from the model grass *Brachypodium distachyon* (BdSQE1) was also expressed in the yeast lines. Preliminary results had shown that this epoxidase was not able to utilize squalene as efficiently in yeast as the

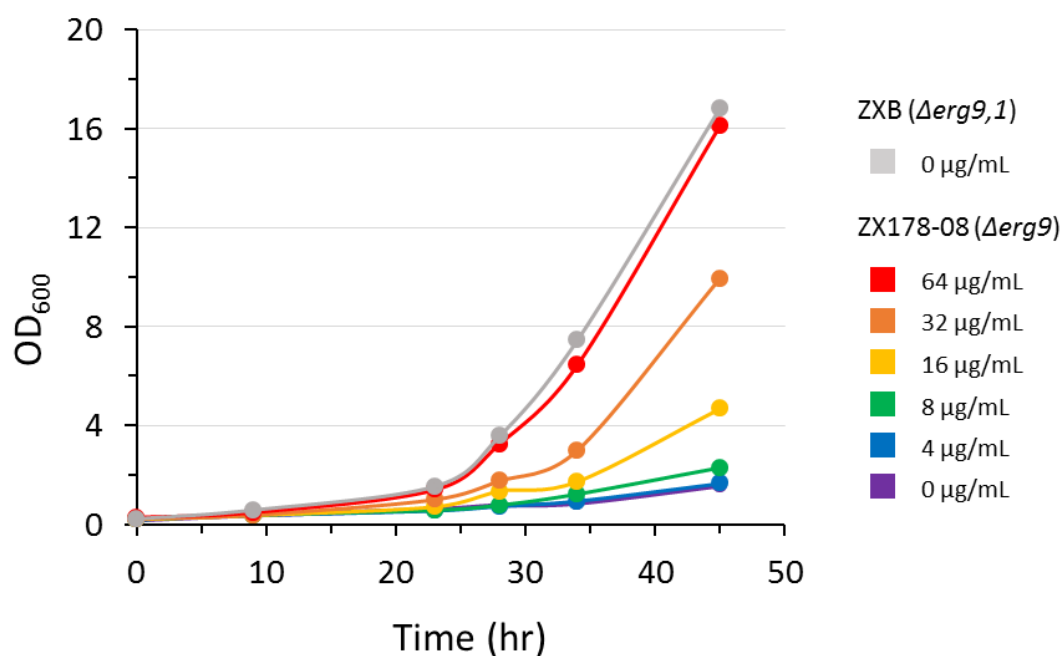


Figure 4.8: Growth of the yeast line ZX178-08 expressing an algal SQS improves with increasing levels of terbinafine. Yeast line ZX178-08 ($\Delta erg9$) was transformed with an algal SQS gene under the control of the galactose-inducible promoter. The ZXB yeast line ($\Delta erg9,1$) was transformed with with the empty expression vector. The resulting transformants were grown in SCE glucose media to stationary phase, and these starting cultures were used to inoculate cultures containing induction media (SCE galactose with varying concentrations of terbinafine. Growth was analyzed at the indicated time points by measurement of the cultures optical density (OD_{600}).

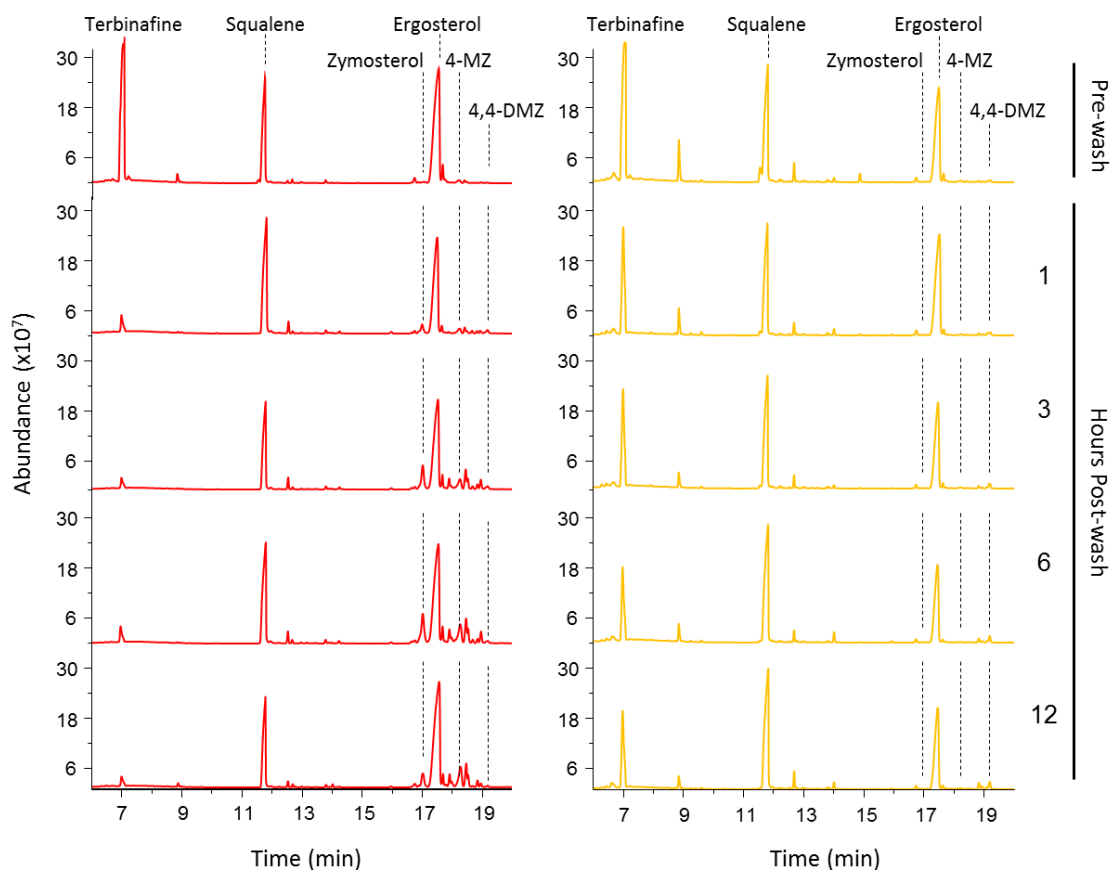


Figure 4.9: Removal of terbinafine from ZX178-08 yeast expressing a human SQS leads to 4,4-dimethylzymosterol accumulation. Yeast line ZX178-08 ($\Delta erg9$) was transformed with an *S. cerevisiae* or *H. sapiens* SQS gene under the control of a galactose-inducible promoter. Cultures were grown in the presence of 64 mg/mL terbinafine in induction media containing ergosterol (SCE galactose) until mid-exponential phase of growth before cells were then washed with sterile water, resuspended in induction media (SCE galactose) and incubation continued. Cells were collected every three hours, saponified and derivatized heptane extracts were analyzed using GC-MS. 4-MZ = 4-methylzymosterol; 4,4-DMZ = 4,4-dimethylzymosterol.

S. cerevisiae squalene epoxidase. We reasoned that expression of this enzyme in tandem with the various *SQS* genes in yeast line $\Delta erg9,1$ would temper the accumulation of the toxin, possibly allowing for a greater chance to visualize toxin accumulation by GC-MS. The $\Delta erg9,1$ yeast line transformed with *BdSQE1* as well as a *SQS* gene from each kingdom of life were grown for three days in induction media before analyzing sterol profiles by GC-MS (Fig. 4.10). Yeast expressing the *S. cerevisiae* *SQS* grew to stationary phase and accumulated each of the expected sterol intermediates, expression of non-fungal *SQS* genes again led to the accumulation of lanosterol and 4,4-dimethylzymosterol. While there is no precedence for 4,4-dimethylzymosterol inhibiting yeast growth [71,72], both of these intermediates are methylated at the C4 position suggesting that the cells expressing a non-fungal *SQS* gene were unable to demethylate the C4 methylated intermediates.

Sterol C4-decarboxylase expression enables a non-fungal SQS to complement a $\Delta erg9$ mutation in yeast

Although an unusual or toxic sterol intermediate was not identified in the foregoing experiments using terbinafine or a poorly active squalene epoxidase, they hinted at a limitation of the C4 demethylation complex in those lines not expressing a fungal *SQS* gene. To evaluate this possibility, enzymes in the C4 demethylation complex were over-expressed in an attempt to overcome this putative metabolic block. Each of the three *S. cerevisiae* genes involved in this complex were PCR amplified and inserted behind a galactose-inducible promoter, transformed into the ZXB yeast line ($\Delta erg9,1$) and three independent transformants plated onto induction media containing either ergosterol or lanosterol (Fig. 4.11). Expression of genes corresponding to the first and third steps of this complex, the methyloxidase (*ERG25*) and ketoreductase (*ERG27*), did not allow the yeast line to grow in the presence of lanosterol. However, yeast expressing the gene for the *S. cerevisiae* sterol C4-decarboxylase (*ERG26*) were able to grow and mimicked the growth phenotype observed when expressing a fungal *SQS*.

Since increased levels of the yeast sterol C4-decarboxylase rescued growth of the *SQS* knockout yeast line and prior work demonstrated the importance of the C-terminal domain of fungal *SQS* in the complementation phenotype, it was reasonable to examine whether or not the C-terminal domain of the fungal *SQS* could mediate the efficiency of the sterol C4 demethylation steps. If so, a hypothesis would be that induced expression of the yeast *ERG26* gene would be able to rescue growth of yeast expressing a non-fungal *SQS*. Furthermore, if the

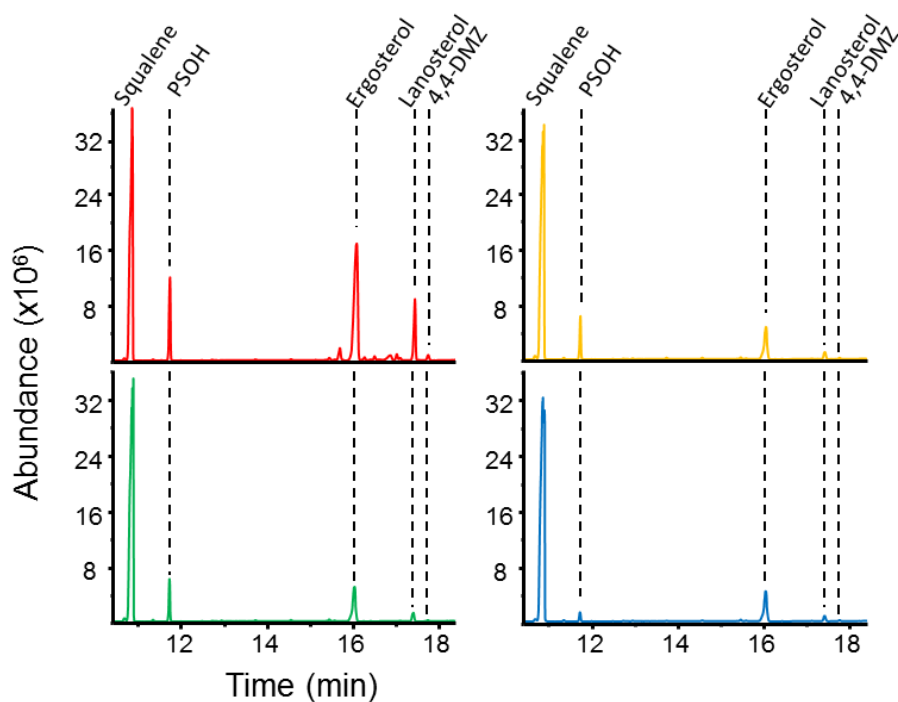


Figure 4.10: Co-expression of a non-fungal SQS with squalene epoxidase from a model grass in ZXB leads to 4,4-dimethylzymosterol. Yeast line ZXB ($\Delta erg9,1$) was transformed with a SQS gene from a yeast, *S. cerevisiae* (red); plant, *A. thaliana* (green); human, *H. sapiens* (yellow); or alga, *B. braunii* (blue) under the control of a galactose-inducible promoter as well as a galactose-inducible squalene epoxidase gene (*ERG1*) from the model grass *B. distachyon*. Yeast cultures were grown first in SCE glucose media and induced by centrifuging the cultures and resuspending the cell pellets in SCE galactose media. Saponified and derivatized sterol extracts were analyzed by GC-MS. 4,4-DMZ = 4,4-dimethylzymosterol.

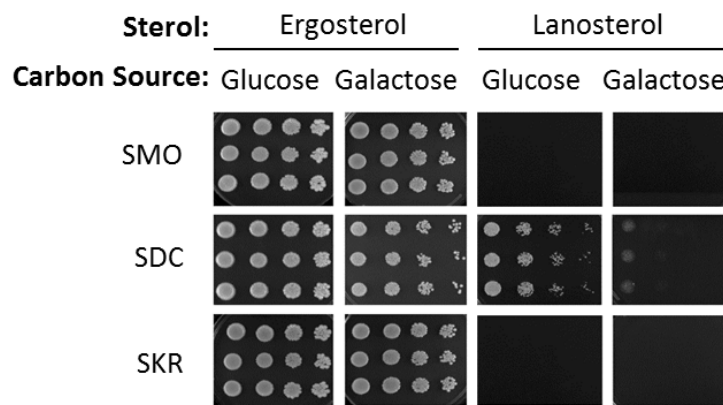


Figure 4.11: Overexpression of sterol C4-decarboxylase allows $\Delta erg9,1$ yeast to grow in the presence of exogenous lanosterol. Yeast line ZXB ($\Delta erg9,1$) was transformed with genes from the yeast *S. cerevisiae* responsible for C4 demethylation, sterol C4-methyloxidase (*ERG25*, SMO), sterol C4-decarboxylase (*ERG26*, SDC), and sterol C3-ketoreductase (*ERG27*, SKR), under the control of a galactose-inducible promoter. Three independent transformants representing each construct were grown in non-inducing media (SCE glucose), density normalized, five-fold serial diluted, and plated on glucose and galactose media containing either ergosterol or lanosterol.

yeast SQS is involved in a fungal-specific protein-protein interaction with a yeast C4-decarboxylase, then it would follow that the plant C4-decarboxylase would specifically detoxify yeast expressing the plant SQS, or the human C4-decarboxylase would recover growth of yeast expressing the human SQS. Plant, human, and algal genes for sterol C4-decarboxylase were PCR amplified, verified by DNA sequencing (Fig. 4.12) and subsequently co-transformed into the ZX178-08 ($\Delta erg9$) yeast line with expression vectors containing genes for SQS and sterol C4-decarboxylase expression. Three independent transformants from each combination were first grown to stationary phase in SCE glucose, then serial dilutions were spotted on media for either low or high level expression with and without exogenous ergosterol. Contrary to our expectations, expression of any sterol C4-decarboxylase gene was able to restore the growth phenotype regardless of the co-expressed SQS gene (Fig. 4.13).

Yeast co-expressing a fungal C4-decarboxylase and a SQS from any kingdom of life were also examined for their sterol profile. Cultures were first initiated in SCE glucose, then switched to induction media (SCE galactose) and induced for an additional three days. Non-saponifiable lipids were extracted and derivatized samples examined by GC-MS (Fig. 4.14). Most striking was that the total sterol profiles for all of the different lines were nearly identical to one another and to the capitulation of the fungal SQS gene with the fungal C4-decarboxylase gene, regardless of the SQS gene they harbored and as long as they co-expressed the fungal C4-decarboxylase gene.

When expressing a yeast C4-decarboxylase from *S. cerevisiae*, the growth phenotypes for fungal and non-fungal SQS expression on glucose containing media were unchanged. Those expressing the yeast SQS grew well while yeast expressing a non-fungal SQS displayed an impaired growth phenotype. Once induced, however, the yeast C4-decarboxylase was able to rescue growth of yeast over-expressing a non-fungal SQS. Just like induced co-expression of both the active and inactive forms of a fungal SQS in figure 3.14, high level co-expression of the yeast SQS along with the yeast C4-decarboxylase impaired growth of the $\Delta erg9$ yeast line. Interestingly, sterol profiles confirmed that the sterol biosynthetic potential was functionally equivalent whether the sterol C4-decarboxylase was over-expressed with a fungal or non-fungal SQS. Co-expression of a plant, human, or algal C4-decarboxylase was able to restore the wild-type growth phenotype in all media types no matter the origin of the SQS. Altogether, these results point to some interruption of the sterol demethylation complex as a result of heterologous expression of SQS genes in the *ERG9* mutant yeast and that either over-

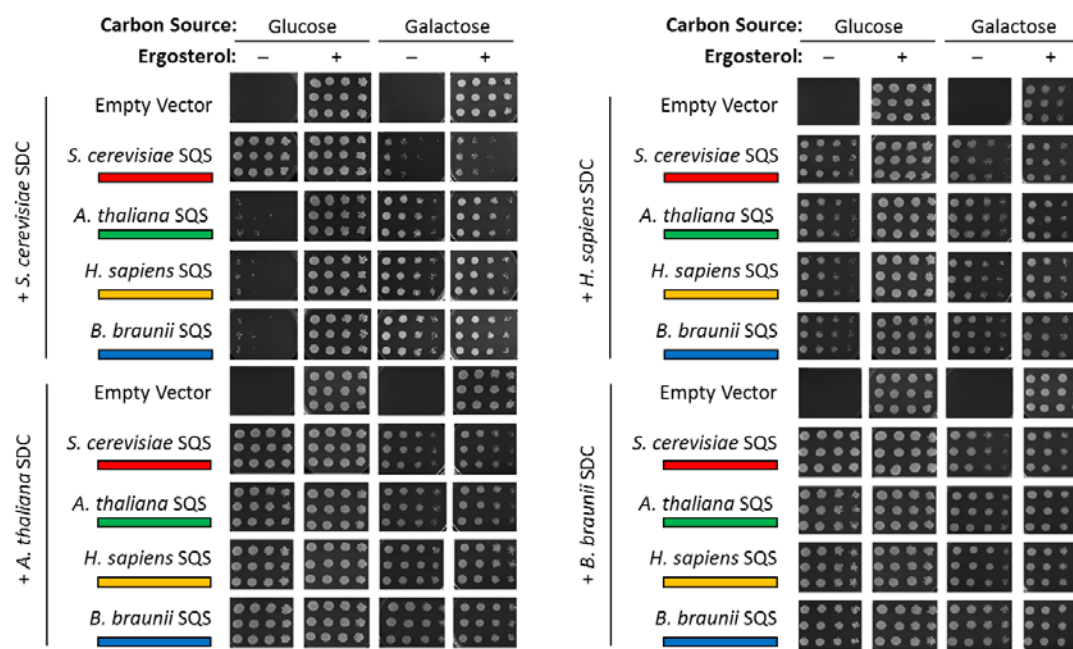


Figure 4.13: Co-expression of sterol C4-decarboxylase genes from any kingdom of life with a SQS gene from any kingdom of life is sufficient to complement a $\Delta erg9$ mutation in yeast regardless of strong (galactose) or leaky (glucose) gene expression conditions. The sterol C4-decarboxylases genes (*ERG26*, *SDC*) from each kingdom of life were transformed with an empty expression vector or a *SQS* gene from a yeast, *S. cerevisiae* (red); plant, *A. thaliana* (green); human, *H. sapiens* (yellow); or alga, *B. braunii* (blue). Independent transformants (rows on each plate) were five-fold serial diluted three times (columns) and spotted onto each media type. Plates were grown for 7 days at 28°C before imaging.

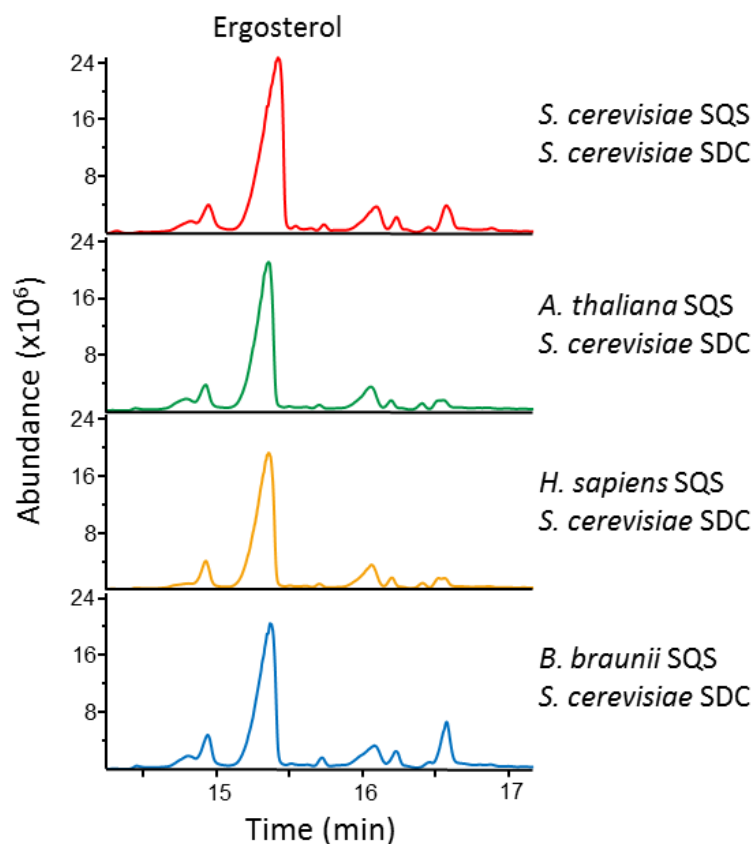


Figure 4.14: Co-expression of a non-fungal SQS with the *S. cerevisiae* sterol C4-decarboxylase returns the sterol pathway to a wild-type profile. Yeast line ZX178-08 ($\Delta erg9$) was transformed with galactose-inducible SQS genes from *S. cerevisiae* (red), *A. thaliana* (green), *H. sapiens* (yellow), and *B. braunii* (blue) in combination with the *S. cerevisiae* sterol C4-decarboxylase gene. Cultures were grown in non-inducing media (SCE glucose), centrifuged and the cell pellets resuspended in induction media (SCE galactose). After three days of further incubation, culture densities were normalized and cells were saponified followed by heptane extraction. The non-saponifiable sterols were derivatized and analyzed by GC-MS.

expression of a fungal or non-fungal C4-decarboxylase suffices to complement this deficiency. This complementation may arise because the heterologous C4-decarboxylases are more active in yeast than the native yeast C4-decarboxylase or because they are not subject to the same regulation as the native yeast C4-decarboxylase.

Expression of a non-fungal SQS leads to the production of carboxysterol intermediates

The disruption of sterol C4-decarboxylase activity using either a full knockout mutation of the *ERG26* gene [67] or a temperature-sensitive mutation [73] was shown to disrupt cell growth and led to the accumulation of toxic carboxysterol intermediates [67]. To determine if such intermediates could be accumulating under some of the conditions described here, it was necessary to adjust the sterol extraction procedure. We grew the $\Delta erg9$ yeast line transformed with SQS genes from each kingdom of life in SCE glucose to mid-exponential phase and induced expression with SCE galactose for three days. Cultures were normalized *via* optical density before performing a Bligh and Dyer total lipid extraction using chloroform and methanol in the presence of acid-washed glass beads. Figure 4.15 shows the resulting chromatograms from GC-MS analysis of derivatized lipids at $m/z = 468$ which highlights both the changes in ergosterol levels and the presence or absence of carboxysterol intermediates.

Yeast transformed with an empty vector accumulated low levels of ergosterol from the growth media, and there was no evidence of any additional sterol intermediates. Following expression of a yeast SQS from *S. cerevisiae* or *C. albicans*, the level of ergosterol increased significantly without any detectable carboxysterol intermediates. When expressing a non-fungal SQS, the level of ergosterol did not increase beyond that which was observed in yeast containing the empty vector. This finding suggested that the yeast were unable to produce significant amounts of ergosterol. Instead, GC analysis revealed two new lipid peaks. These two peaks were also present following expression of a non-fungal SQS when the promoter was repressed and no exogenous ergosterol was present (SC glucose). That suggested that the impaired growth observed in this media occurred by the same mechanism as growth inhibition following induction (Fig. 4.16). Using the MS data obtained from these studies (Fig. 4.17) in comparison to a commercial vendor's report for carboxy sterols [74], we tentatively identified the peaks as trimethylsilylated forms of a 4-methyl-4-carboxy-bisunsaturated sterol. Since the parent ion ($m/z = 586$) was conserved between these two peaks, we suspected that these two compounds were epimers of one another.

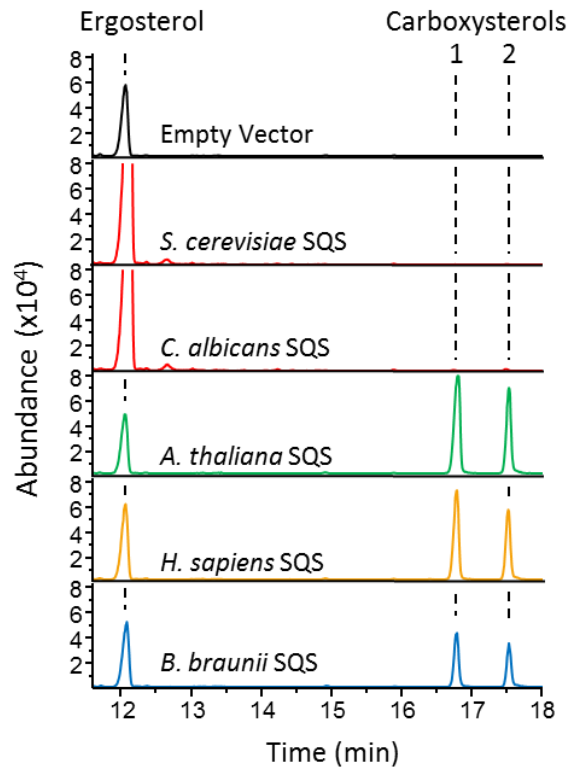


Figure 4.15: Overexpression of a non-fungal SQS gene leads to accumulation of carboxy-sterol intermediates. Yeast line ZX178-08 ($\Delta erg9$) was transformed with an expression vector containing a galactose-inducible SQS gene from a yeast, *S. cerevisiae* or *C. albicans*; plant, *A. thaliana* (green); human, *H. sapiens* (yellow); or alga, *B. braunii* race B (blue). Cultures were grown in SCE glucose, centrifuged, and the cell pellets were resuspended in SCE galactose induction media. After three days, cultures were density normalized; total lipids were extracted, derivatized, and analyzed by GC-MS. In order to enhance visualization of the sterol metabolites, selective ion monitoring was used ($m/z=468$).

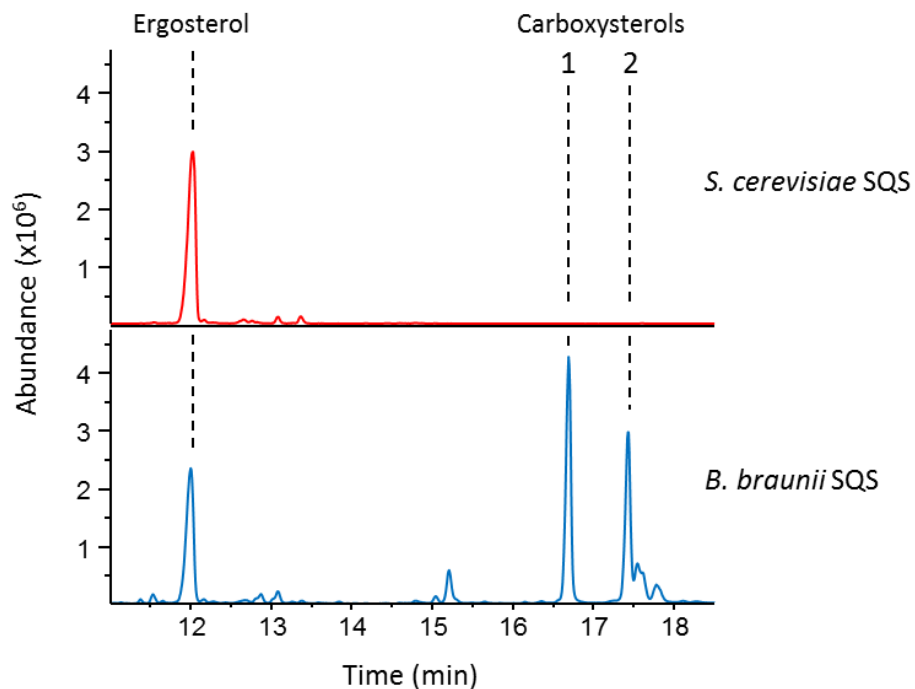


Figure 4.16: Low level expression of a non-fungal SQS led to accumulation of carboxy-sterol intermediates. Yeast line ZX178-08 ($\Delta erg9$) was transformed with yeast *S. cerevisiae* (red) and algal *B. braunii* race B (blue) SQS genes under the control of a galactose-inducible promoter. Cultures were initially grown to stationary phase in non-inducing media with ergosterol (SCE glucose), centrifuged, and the cell pellets resuspended in the same media lacking a source of exogenous sterols (SC glucose). Total lipids were extracted and derivatized for GC-MS analysis.

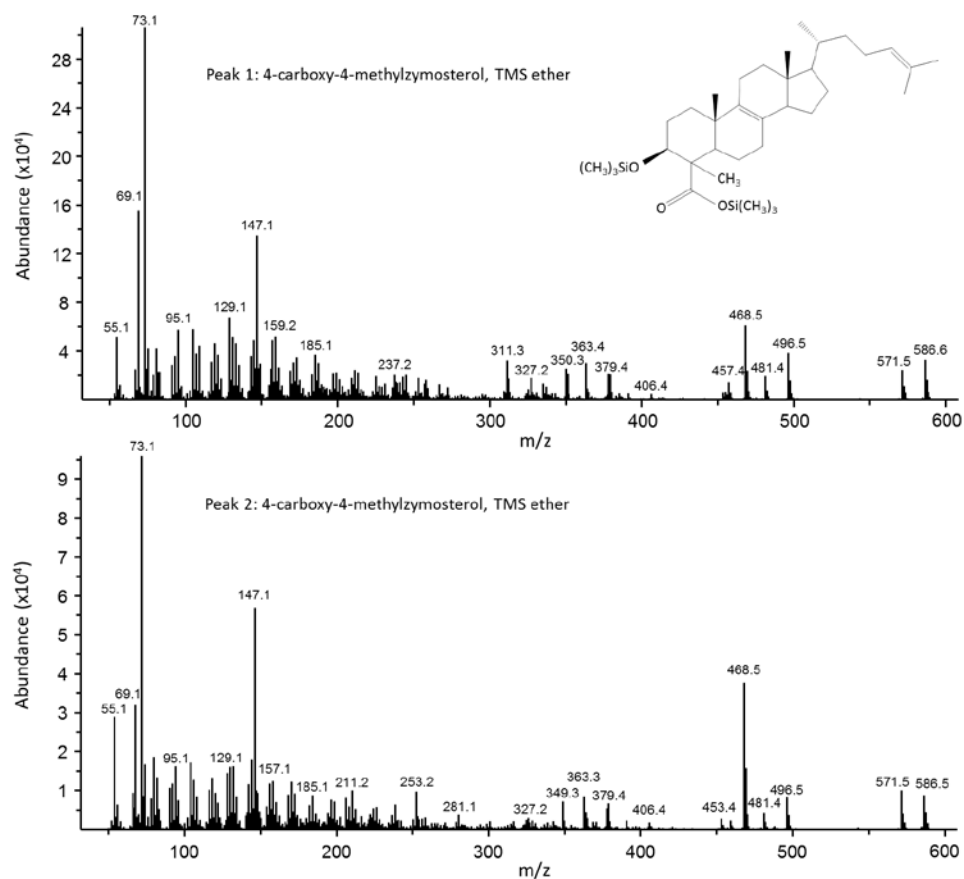


Figure 4.17: Mass spectra of putative carboxysterol intermediates. The MS patterns for peaks with retention times 16.68 and 17.38 minutes in figure 4.16.

In order to further support our claim that the two peaks are carboxysterols, we profiled the sterol pathway of yeast grown in the presence of totarol, a compound isolated from the *Podocarpus totara* tree in New Zealand previously shown to inhibit the sterol C4-decarboxylase protein in yeast [75]. The wild-type yeast line BY4741 was incubated for three days with 150 ng/mL of totarol which led to a 25% growth inhibition. Following total lipid extraction, derivatized sterols were examined by GC-MS. Not only were the same two carboxysterol peaks present in the totarol treated yeast extracts, an additional peak with a mass spectrum consistent with a 4-carboxy-bisunsaturated sterol was observed (Fig. 4.18A-B).

All together, these results suggest that while totarol only partially inhibits the sterol C4-decarboxylase in yeast, lack of a fungal SQS C-terminal domain is far more disruptive to the demethylation step leading to almost complete growth inhibition. It is also plausible that SQS is responsible for controlling the first decarboxylation step, but the second step is not inhibited, which would also account for the absence of 4-methylzymosterol in the total lipid extractions (Fig. 4.18C). Regardless, additional studies such as NMR will be required to verify the identity of these two carboxysterol intermediates (as well as the position of the double bonds and stereochemistry of the 4-carboxy group) accumulating in the $\Delta erg9$ yeast lines expressing non-fungal SQS genes.

As another method to confirm the role of these two putative carboxysterol peaks in yeast growth inhibition, we co-transformed the *B. braunii* race B SQS gene with a sterol C4-decarboxylase gene (*ERG26*) from each kingdom of life or the mutant yeast SQS gene encoding for an inactive SQS enzyme. Growth of the transformed lines was compared, as were the levels of the putative carboxysterols. Each of these genes detoxified the yeast expressing a non-fungal SQS, returning a near or actual wild-type growth phenotype. We therefore expected the resulting carboxysterol peaks from these combinations to be eliminated or at least greatly decreased in comparison to yeast expressing the non-fungal SQS alone. While co-expression with the inactive yeast SQS or non-fungal *ERG26* genes decreased the total carboxysterol level by more than 82%, co-expression of the algal SQS gene with the gene encoding the *S. cerevisiae* C4-decarboxylase only decreased the total carboxysterols by 27% (Fig. 4.19). Yet, growth of this line was near normal under induction conditions (galactose). It is conceivable that this was due to the carboxysterol accumulating during growth under the non-inducing conditions (SCE glucose), since the fungal C4-decarboxylase did not allow the non-fungal SQS to complement fully the $\Delta erg9$ mutation when the promoter was repressed (Fig. 4.13).

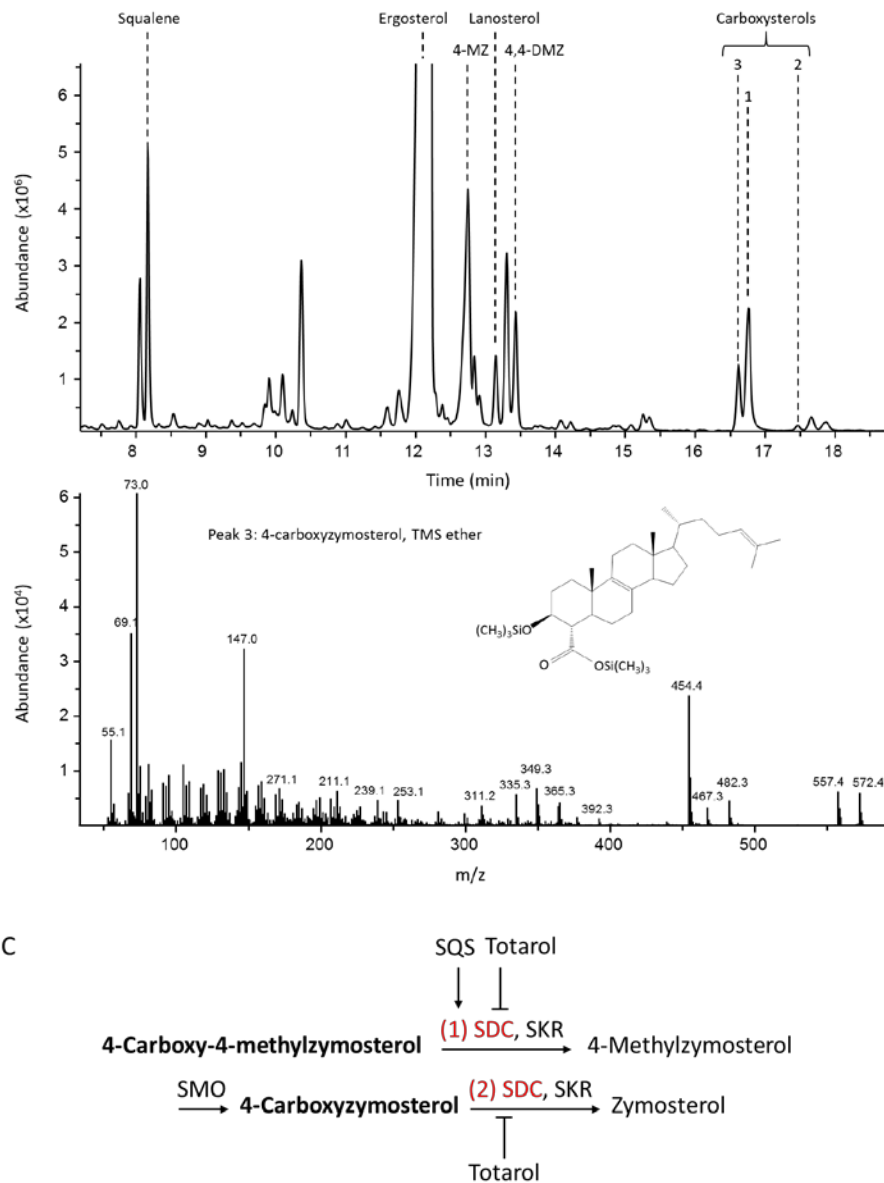


Figure 4.18: Accumulation of three carboxysterol intermediates in wild-type yeast following totarol treatment. Wild-type yeast line BY4741 was grown in complete media (YPD) with 150 ng/mL totarol. Total lipids were extracted and derivatized for GC-MS analysis. A) Peaks 1 and 2 of the total lipid profile correspond to the previously identified carboxysterols. B) The mass spectrum of the carboxysterol identified from peak 3. C) A comparison of the function a fungal SQS may have in the C4 demethylation complex compared to totarol treatment. The two enzymatic steps involving the sterol C4-decarboxylase (SDC) are numbered.

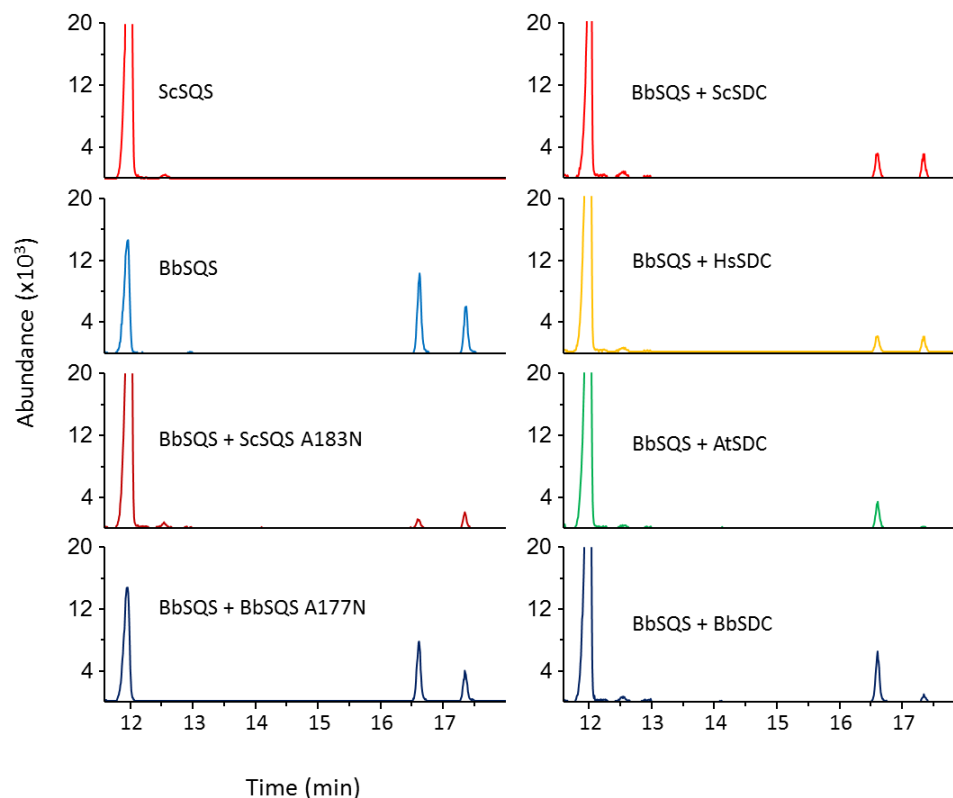


Figure 4.19: Yeast expressing a non-fungal SQS have lower total carboxysterol levels when co-expressing an inactive yeast SQS or a C4-decarboxylase. Yeast line ZX178-08 ($\Delta erg9$) expressing an algal SQS from *B. braunii* race B (BbSQS) were co-transformed with an expression vector containing an inactive yeast SQS gene with an A183N mutation, an inactive algal SQS gene with an A177N mutation, or a sterol C4-decarboxylase gene (*ERG26*, *SDC*) from yeast (ScSDC), human (HsSDC), plant (AtSDC), or algal (BbSDC) cells. Cultures were grown to mid-exponential phase and induced for three days. Total lipids were extracted, derivatized, and analyzed by GC-MS using selective ion monitoring at $m/z = 468$.

Discussion

It has been suggested that the C-terminal domain of SQS is responsible for either stabilizing the enzyme and preventing its degradation *in vivo*, or localizing it to the ER membrane [39]. While this domain contains the membrane spanning helix, we determined that fungal SQS proteins are required to prevent the accumulation of a toxic sterol. This work furthers these findings by demonstrating that growth inhibition caused by expression of a non-fungal SQS can be prevented by overexpressing a sterol C4-decarboxylase from any kingdom of life. This led to a decrease in the accumulation of two carboxysterol intermediates. These findings suggest that the mechanism for the C-terminal domain of SQS involves proper functioning of the yeast sterol C4-decarboxylase.

Much like 3-ketosterols that destabilize the lipid bilayer and increase permeability of erythrocyte membranes, the accumulation of carboxysterols could be toxic due to changes in membrane fluidity and permeability [76]. In *S. cerevisiae*, accumulation of carboxysterol intermediates is accompanied by a dramatic decrease in growth as well as defects in the biosynthesis of membrane components including phosphatidic acid, phosphatidylinositol and mono-, di-, and triglycerides [67,73]. In animal cells, defects in the C4-decarboxylase protein (NSDHL) are associated with X-linked, male-lethal phenotypes in both mice and humans. While mice develop phenotypes including bare patches (Bpa) and striated (Str), C4-decarboxylase mutations in humans lead to CHILD syndrome, a developmental disorder characterized by hemidysplasia, ichthyosiform erythroderma and limb defects [77,78]. Even plant growth is negatively affected by accumulating carboxysterol intermediates, which disrupt polar auxin transport and root development [79].

While the C-terminal domain of SQS may not regulate stability or localization of SQS it may influence assembly of C4-decarboxylase into complexes affecting enzyme activity and protein stability. Teske *et al.* observed that the sterol C3-ketoreductase in yeast also has a regulatory mechanism which is separate from its catalytic activity, and may be required for proper folding and conformation of the lanosterol synthase (Erg7) enzyme. Even when the lanosterol synthase properly localized to the ER membrane, the ketoreductase was required for its activity, and the interaction between these two proteins was shown to be evolutionarily conserved among fungal sterol pathways. Likewise, SQS may be required to chaperone the yeast C4-decarboxylase. This could explain why the yeast C4-decarboxylase was unable to fully rescue growth of yeast expressing a non-fungal SQS without galactose induction. The expressed

protein would have been too unstable, while the non-fungal C4-decarboxylase proteins would not necessarily be subject to the same regulation in yeast.

While the yeast C4-decarboxylase does not contain a recognizable ER retrieval signal, studies have shown that this protein remains tethered to the ER membrane. This is unlike many other proteins in the sterol pathway that localize both to the ER and either lipid particles or vesicles via recognizable membrane spanning motifs [80]. The C4-decarboxylase may, therefore, require another protein to act as a tether to the ER membrane. Studies from the Bard laboratory have suggested that members of the C4 demethylation complex, either the sterol C4-methyloxidase (SMO, Erg25) protein or the Erg28 protein, may carry out this role as the former has a C-terminal ER retrieval signal and the latter acts as a scaffold protein for the complex [62,71]. While the Erg28 protein has been shown to interact with the C4-methyloxidase, C4-decarboxylase, and C3-ketoreductase through yeast-2-hybrid analysis and co-immunoprecipitation, it is possible that the yeast SQS may be more critical for regulating SDC localization in yeast [81,82]. Unlike in yeast, the plant, human, and algal C4-decarboxylase proteins all have C-terminal ER localization signals, which may also explain why these proteins are able to recover fully the growth of yeast expressing a non-fungal SQS even without galactose induction.

Chapter 5: Mapping a Kingdom-Specific Hinge Motif in the C-terminal Domain of Squalene Synthase

Introduction

Conventional drug design has focused primarily on inhibiting a protein, usually an enzyme or receptor, by targeting its active site or ligand-binding site. In contrast, the inhibition of protein-protein interactions involved in many important cellular processes is an emerging area of drug design [83]. Protein-protein interactions are often categorized in two types: stable and transient. Stable interactions are usually involved in creating macromolecular complexes such as the three enzymes that form RNA polymerase II, or likely the sterol C4 demethylation complex, and the interactions occur between domains [84]. Alternatively, transient interactions often mediate signaling and regulation processes. These transient interactions generally occur between a short recognition motif, often a helix, which binds into a small groove in a relatively flat recognition domain [85].

While developing drugs that target these interactions has proven challenging, there are some promising approaches [83]. Strategies include the production of large and complex drug molecules to fit the large interfaces between proteins or designing small molecules that bind weakly throughout the interface and then combining them to create a single lead with high affinity [86]. Perhaps the most successful method to date has been the development of small molecules and peptidomimetics that resemble the peptide motif involved in domain-motif interactions. The interaction between p53 and its E3-ubiquitin ligase MDM2 has been one such targeted interaction with small molecule inhibitors currently in different phases of clinical trials [87]. These anticancer agents mimic a helix of p53 in order to target the binding cleft on MDM2.

While peptides are limited in their ability to work as therapeutic agents by susceptibility to proteolysis and poor cell-penetrability, peptidomimetic agents involving non-canonical amino acids, stapling of helical conformations and cyclization are more promising [88,89]. For example, multiple laboratories are currently working on developing and testing compounds capable of mimicking the peptide motif of the protein second mitochondrial-derived activator of caspases (Smac) which binds to the BIR domains of carboxy-linked inhibitor of apoptosis protein (XIAP). In so doing, they have been able to disrupt XIAP and release caspases to cause apoptosis of cancer cells [90,91].

While it is not yet clear how SQS is involved in disrupting the fungal demethylation complex (see Chapter 4), it is quite likely that this phenotype involves a protein-protein interaction of some kind. An analysis of the sterol biosynthetic complex using yeast 2-hybrid technology identified multiple binding partners for the yeast SQS, but only showed a weak interaction between SQS and the C4-decarboxylase [81]. This may mean that the interaction is transient or SQS has an effect on the C4-decarboxylase indirectly through other binding partners. The aim of the work reported in this chapter was to identify the motif in the C-terminal domain of *S. cerevisiae* SQS responsible for the growth inhibition phenotype and to test whether or not expression of the motif is able to disrupt an interaction and lead to yeast growth inhibition.

Results

Mapping a functional motif in the C-terminal domain of SQS

To identify the structural feature(s) of SQS responsible for mediating the toxicity phenotype, we constructed chimeric genes and tested for their ability to complement the SQS knockout mutation. Since Kribii *et al.* determined that the C-terminal 110 amino acids of the SQS from fission yeast *S. pombe* is enough for a plant SQS to complement the $\Delta erg9$ mutation in yeast, we identified the corresponding region in the SQS genes from each kingdom of life and swapped these C-terminal domains in reciprocal fashion. We transformed the yeast line ZX178-08 with each chimeric gene, grew three independent transformants for each, serially diluted and spot plated the yeast on various media types (Fig. 5.1). As expected, genes harboring the fungal C-terminal domain mimicked the phenotype of yeast expressing a full-length fungal SQS and grew in the tested conditions regardless of the source gene for the amino-terminal catalytic domain. Those harboring any of the non-fungal C-terminal domains exhibited a limited ability to complement the $\Delta erg9$ mutation when the promoter was repressed, and inhibited growth when induced.

Since the complementation phenotype is specific to the fungal C-terminal domains, we aligned this region of multiple fungal SQS enzymes from *S. cerevisiae*, *C. albicans*, *A. nidulans*, and *S. pombe* and found only 8.3% identity between the sequences (Fig. 5.2). While the membrane spanning helix showed very little conservation, a 26-amino acid sequence proximal

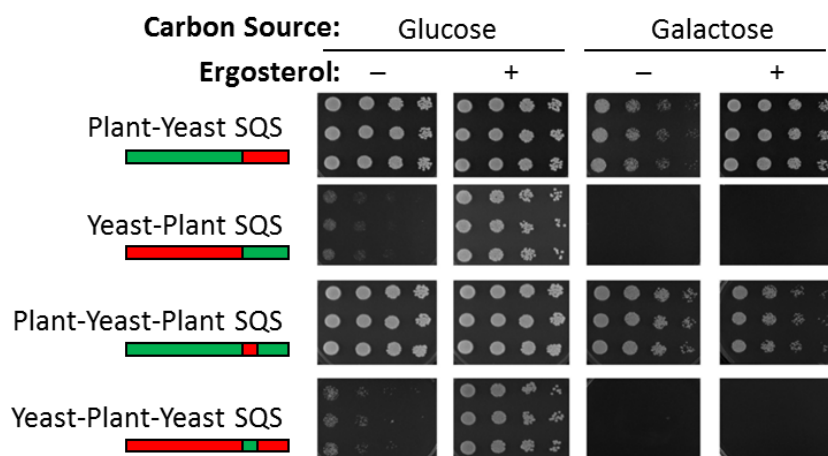


Figure 5.1: Mapping the SQS motif responsible for the functional complementation phenotype. Chimeric SQS genes were created by reciprocal exchanges of yeast, *S. cerevisiae* (red), and plant, *A. thaliana* (green) SQS genes. All gene constructs were inserted downstream of the galactose-inducible promoter, the resulting plasmids transformed into yeast line ZX178-08, and three independent transformants serial diluted five-fold onto inducing (galactose) and non-inducing (glucose) media, with and without added ergosterol.

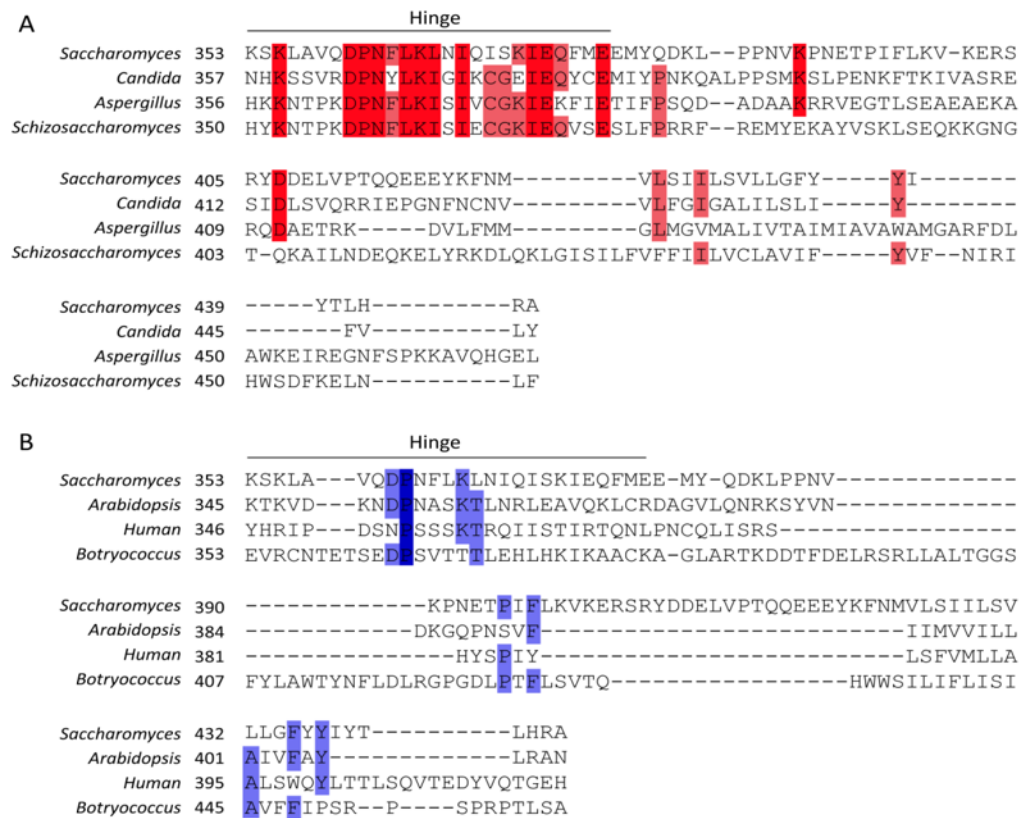


Figure 5.2: Alignments of the C-terminal domains of SQS enzymes from diverse fungi and from multiple kingdoms of life. The C-terminal domains of SQS corresponding to the 110 C-terminal amino acids shown by Kribii *et al.* to be responsible for the kingdom-specific complementation of the $\Delta erg9$ mutation in yeast were aligned using the clustal omega program. (A) An alignment of fungal SQS C-terminal domains including *S. cerevisiae*, *C. albicans*, *A. nidulans*, and *S. pombe*. (B) An alignment of the C-terminal domains of SQS between kingdoms of life including a fungus, *S. cerevisiae*; plant, *A. thaliana*; animal, *H. sapiens*; and alga, *B. braunii*. These C-terminal domains consist of a membrane spanning domain, preceded by a hinge domain that links the membrane spanning domain to the catalytic domain (not shown).

to the catalytic domain had a sequence identity of 38.5%. We will subsequently refer to this sequence as the “hinge motif.” The amino acid sequence of interest from the *S. cerevisiae* SQS is KSKLAVQDPNFLKLNQISKIEQFME. Of those 26 amino acids, two segments are the best conserved among fungal SQS proteins: DPNFLKL and KIEQ. We then performed an alignment of the C-terminal domains of SQS across multiple kingdoms of life and found the previously identified hinge motif is not conserved between kingdoms other than the amino acids DPN, indicating that the motif may have a fungal-specific function. According to a homology model of SQS, the “hinge motif” appears to reside adjacent to the catalytic domain and is composed of a short helical segment, followed by a loop and longer helix (Fig. 5.3).

In order to evaluate this motif for its ability to convey the fungal-specific complementation phenotype to non-fungal SQS enzymes, we produced chimeric enzymes by reciprocally swapping only the hinge motif. After transforming these constructs into ZX178-08 and spot plating independent transformants, we discovered that each SQS, regardless of its kingdom of origin, was able to complement the knockout mutation when harboring a fungal hinge motif (Fig. 5.1). The reverse was also true, in that each fungal SQS was incapable of fully complementing the $\Delta erg9$ mutation when the sequence contained the 26 amino acids from a non-fungal SQS, and exhibited the toxicity phenotype. GC-MS analysis of transformants harboring the chimeric SQS genes revealed accumulation of the two carboxysterol peaks, suggesting that the cause of toxicity for a fungal SQS with a non-fungal hinge and a full-length non-fungal enzyme was the same (Fig. 5.4).

Further narrowing down the critical residues in the hinge motif

Since small molecule therapeutics function best when mimicking a motif shorter than the full 26-amino acids, we next attempted to narrow this region of interest further. We began by reciprocally swapping a segment of this sequence between the fungal *S. cerevisiae* SQS and the algal SQS from *B. braunii* race B. Since the second helical segment displays the greatest conservation within the fungal kingdom, while the more N-terminal helix only shares a conserved lysine residue, we swapped only the C-terminal 16 amino acids: FLKLNQISKIEQFME in the *S. cerevisiae* SQS and VTTTLEHLHKIKAACK in the *B. braunii* enzyme (Fig. 5.5).

A fungal SQS with the 16 amino acids from the algal hinge motif was no longer able to complement the knockout mutation fully and was phenotypically similar to expression of a non-fungal SQS, suggesting that the second helix was at least partially responsible for the

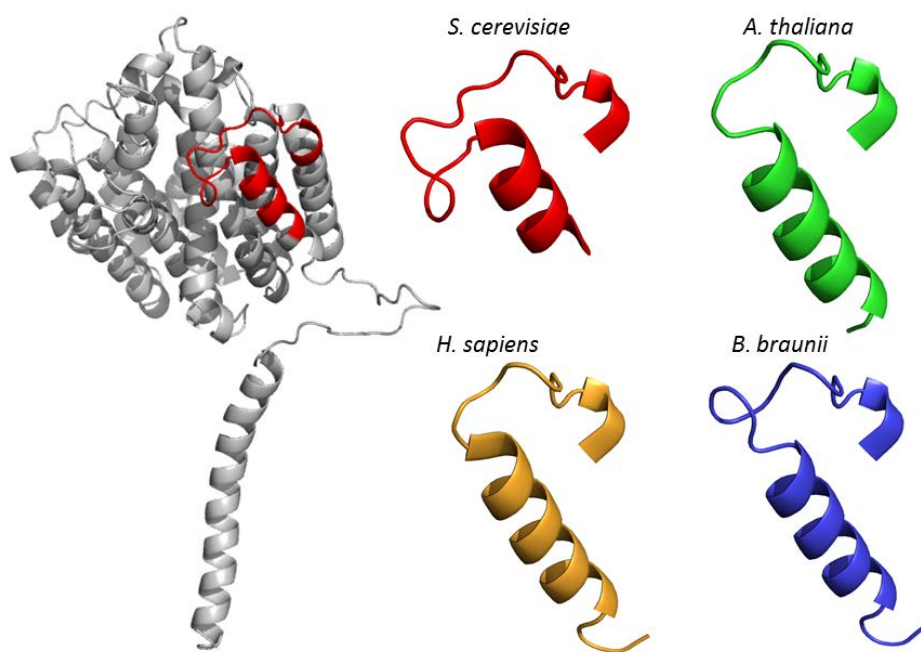


Figure 5.3: A homology model of *S. cerevisiae* SQS and the hinge motifs from each kingdom of life. All homology models were generated using the crystal structure of the human SQS [92]. The hinge motif of the native *S. cerevisiae* SQS (red) is indicated on the homology model (left). Hinge motifs from *S. cerevisiae* (red), *A. thaliana* (green), *H. sapiens* (yellow) and *B. braunii* (blue) SQS are depicted to the right.

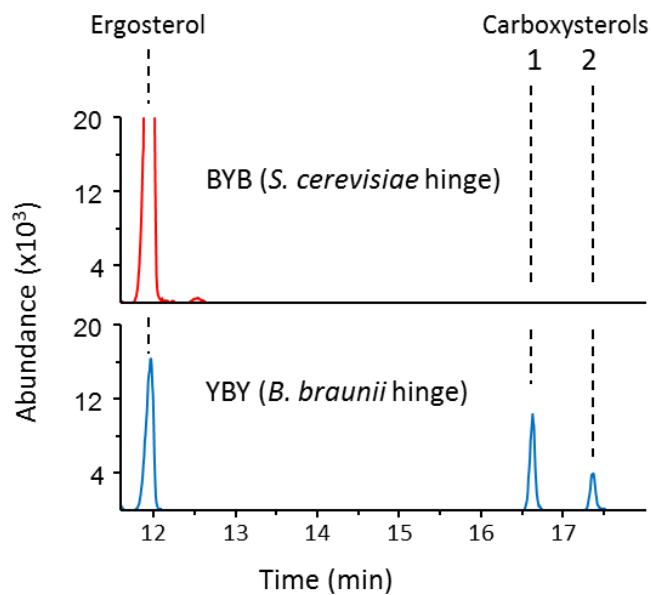


Figure 5.4: Expression of SQS with a non-fungal hinge motif leads to carboxysterol accumulation. Yeast line ZX178-08 ($\Delta erg9$) was transformed with two chimeric SQS genes: one from the alga *B. braunii* harboring a hinge motif from the yeast *S. cerevisiae* (red) and another from yeast with the algal hinge motif (blue). The cells were density normalized and total lipids were derivatized for GC-MS analysis with selective ion monitoring ($m/z=468$).

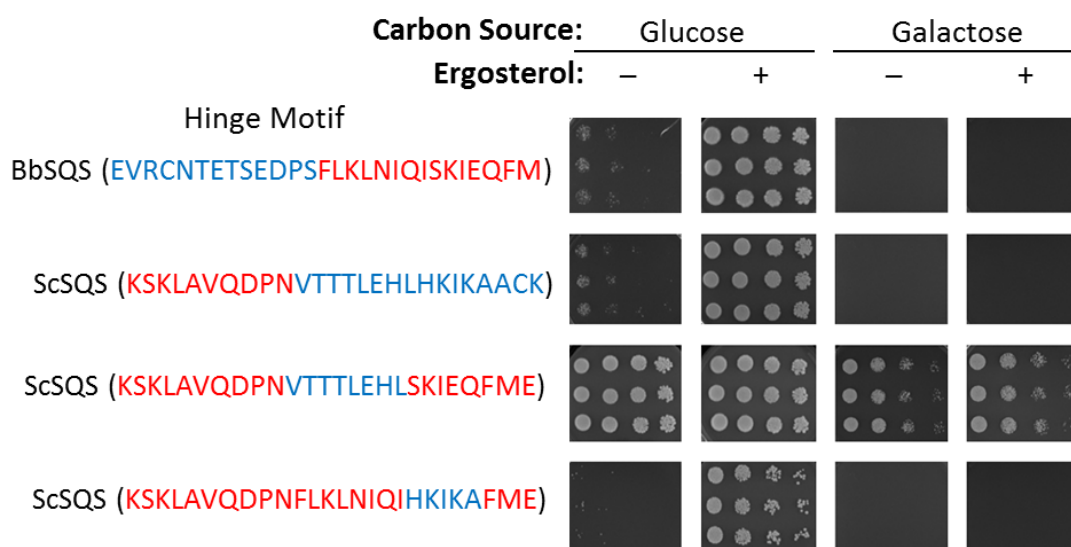


Figure 5.5: The SKIEQ amino acid sequence in the hinge motif is critical for the fungal SQS complementation phenotype. Chimeric SQS genes were created by reciprocally swapping the nucleotide sequence responsible for the final 16 amino acids, producing an algal SQS from *B. braunii* (BbSQS) with the final 16 amino acids of the fungal *S. cerevisiae* hinge motif (red) and the fungal SQS (ScSQS) with the final 16 amino acids of the algal SQS (blue). Chimeric yeast SQS genes were then produced by mutating nucleotide sequences within the second half of the hinge motif to the algal counterpart (blue). The chimeric genes were expressed in ZX178-08 yeast ($\Delta erg9$). Independent transformants (rows) were grown initially in non-inducing media with added ergosterol (SCE glucose), diluted to a normalized density and five-fold serial dilutions were plated on media where the promoter is repressed (glucose) or induced (galactose) with or without ergosterol. Images were taken after seven days of growth at 28 °C.

complementation phenotype. However, the reciprocal was not as expected. An algal SQS with the 16 amino acids from a fungal SQS did not complement the SQS knockout mutation in yeast, suggesting that the initial seven amino acids of the hinge motif are important for an interaction or for proper positioning of the second, well-conserved helix *in vivo*. To further narrow down the critical 16 amino acids of the fungal SQS, we mutated regions within the second half of the *S. cerevisiae* SQS hinge motif to the algal counterpart. Mutation of the first half of the 16 amino acid segment, FLKLNIQI, to the corresponding sequence in the algal hinge motif only marginally decreased the ability of the fungal SQS to complement the knockout mutation, while replacing the final fourth of the fungal hinge motif, SKIEQFME, with the corresponding sequence from the algal SQS led to the toxic growth phenotype. Finally, the region of the fungal hinge motif most critical for the complementation phenotype was narrowed down to the SKIEQ amino acid sequence. Previous work in our laboratory using a constitutive vector system mirrors the results shown here [32].

Additional point mutations were produced in the *S. cerevisiae* SQS hinge motif to identify the contribution of post-translational modifications to the complementation phenotype. Since the C-terminal domain of *S. cerevisiae* SQS has been previously identified as a target for both ubiquitination and sumoylation [93–95], the lysine residues were mutated to arginine both singularly and in combinations (Fig. 5.6). The serine residue was also mutated to cysteine to determine whether phosphorylation was required for complementation. Spot plate analysis suggested that a post-translational modification at these residues was not responsible for the complementation phenotype. Additional mutation of the lysine residues to glutamate showed that the positive charge at these positions was also not required for complementation. Mutating the serine residue alone did not result in any change in yeast growth.

Expression of the fungal SQS hinge motif inhibits growth of wild-type yeast

Since the yeast hinge motif is necessary for a SQS gene to complement the $\Delta erg9$ yeast line and prevent toxicity, we speculated that this amino acid sequence is involved in an essential physical interaction with another protein. Successful inhibitors of this sort of protein-protein interaction have been produced by mimicking the motif of one protein in order to compete for the binding partner. So, we expressed the C-terminal domain of each SQS without the catalytic domain so see if they would out-compete the native yeast SQS for an interacting partner(s). We cloned the C-terminal domains of SQS from various organisms into expression

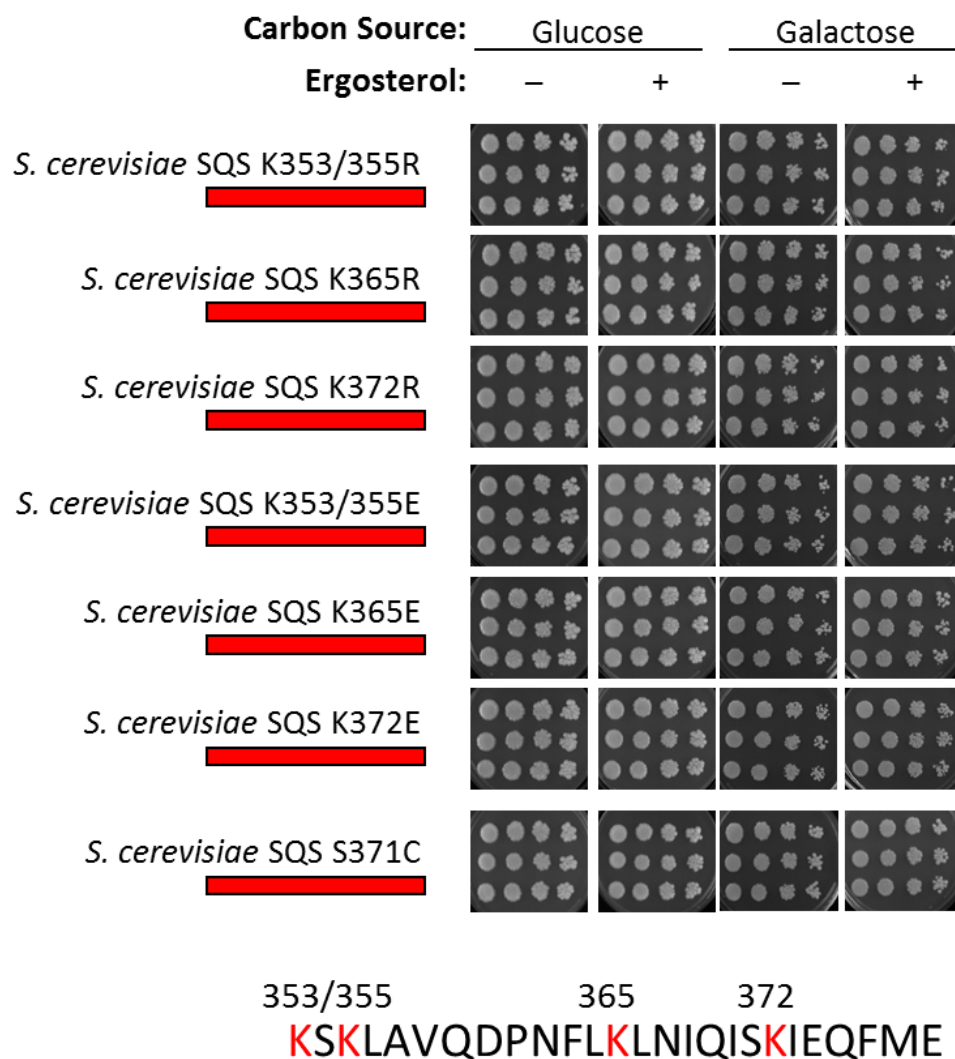


Figure 5.6: Examination of specific residues in the fungal SQS hinge motif for their contribution to the complementation phenotype. Lysine to arginine or glutamate point mutations and a serine to cysteine mutation were introduced to the *S. cerevisiae* SQS gene and the resulting plasmids were transformed into yeast line ZX178-08 ($\Delta erg9$). Three independent transformants were serial dilution plated on to inducing (galactose) and non-inducing (glucose) media, with and without added ergosterol. Yeast cultures were also grown in liquid SCE galactose media and the optical densities were recorded. The amino acid sequence of the hinge motif highlighted for residues mutated is included for clarity.

vectors under the control of the pGAL10 promoter, creating constructs for C-terminal domain expression, and using both the pGAL10 and pGAL1 divergent promoters for two-fold expression. Reciprocal chimeras of the hinge motif and membrane spanning helix were also produced, resulting plasmids were transformed into the wild-type yeast BY4741, and yeast growth was monitored using glucose (repression conditions) and galactose (induction conditions) liquid and solid media over time. As wild-type yeast are not able to use exogenous sterols in aerobic conditions, no ergosterol was added to the growth media.

As observed in the growth curve analysis (Fig. 5.7), none of the expressed C-terminal domains led to a decrease in the growth rate of the wild-type yeast in non-inducing media (SC glucose) compared to the empty vector control. The doubling time for these cultures was approximately 3 hours. Following galactose induction, constructs harboring two copies of a non-fungal C-terminal domain showed no adverse effects of growth relative to the vector control while yeast over-expressing two copies of a fungal SQS C-terminal domain were all dramatically growth impaired. While the doubling time for yeast expressing a non-fungal C-terminal domain under strong inducing conditions was approximately 10 hours, yeast expressing a fungal C-terminal domain had a doubling time of 30 hours. By comparing one-fold and two-fold expression of these domains (Fig. 5.8), it is clear that yeast growth inhibition was dose-dependent. Furthermore, expression of the chimeric C-terminal constructs with various hinge motifs and membrane spanning regions showed that the identity of the hinge region is indeed responsible for this decreased growth performance (Fig. 5.9).

To verify that this growth inhibition occurs *via* the same mechanism as expression of a non-fungal SQS, we profiled the sterols by extracting non-saponifiable lipids or total lipids (Fig. 5.10). The wild-type yeast line BY4741 was transformed with expression vectors harboring two copies of either the *S. cerevisiae* SQS C-terminal domain or the non-fungal SQS C-terminal domains. Yeast were grown in glucose without exogenous ergosterol to repress the promoter, and then induced for three days prior to extraction and analysis. Yeast inhibited by the fungal C-terminal domain displayed a very similar sterol profile when compared to yeast expressing non-fungal C-terminal domains except for a slight increase in lanosterol and decrease in zymosterol accumulation. The total lipid extraction did not reveal an increase in the level of carboxysterol intermediates. As a final method to test the mechanism of growth inhibition, the yeast line ZX178-08 was transformed with the 1x and 2x C-terminal domain expression vectors and spot plated on glucose and galactose media with added ergosterol. If growth inhibition was

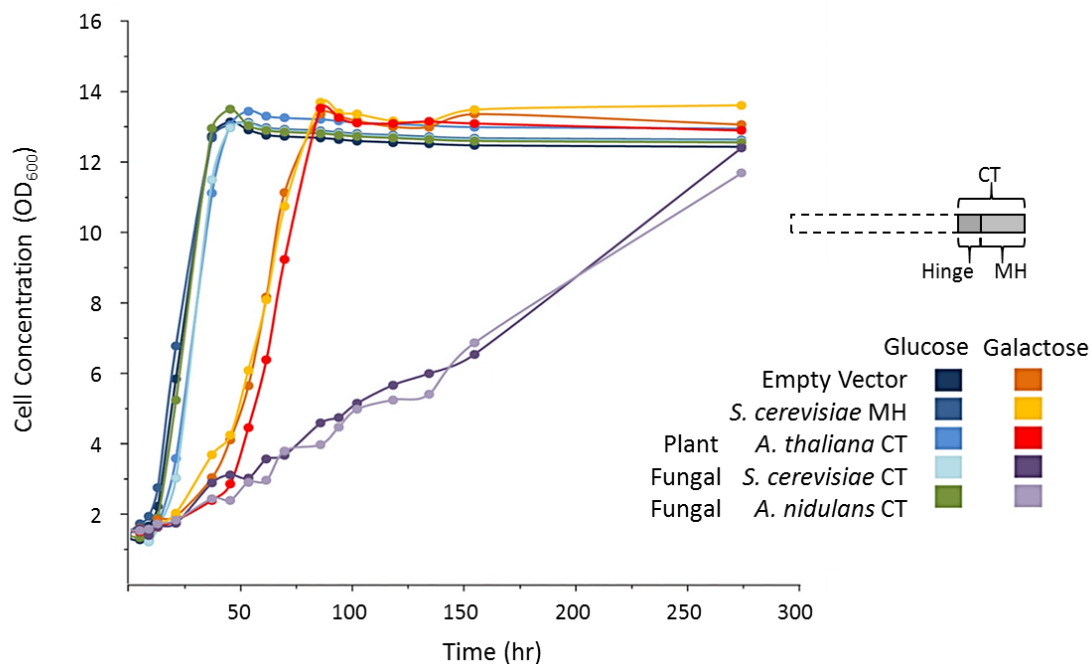


Figure 5.7: Effects of fungal and non-fungal SQS carboxy-terminal (CT) domains on growth of wild-type BY4741 yeast. Expression constructs harboring SQS carboxy-terminal domains comprising the hinge and membrane-spanning domains (without the corresponding amino-terminal catalytic domains) were transformed into wild-type yeast and transformants grown in non-inducing (glucose) and inducing (galactose) media. As an additional control, the yeast SQS membrane-spanning helix (MH) was also tested. Three independent transformants for each construct were grown to stationary phase in glucose media and used to inoculate liquid media containing either glucose or galactose. Growth was monitored as optical density measurements and the standard deviation was never greater than OD₆₀₀=1.

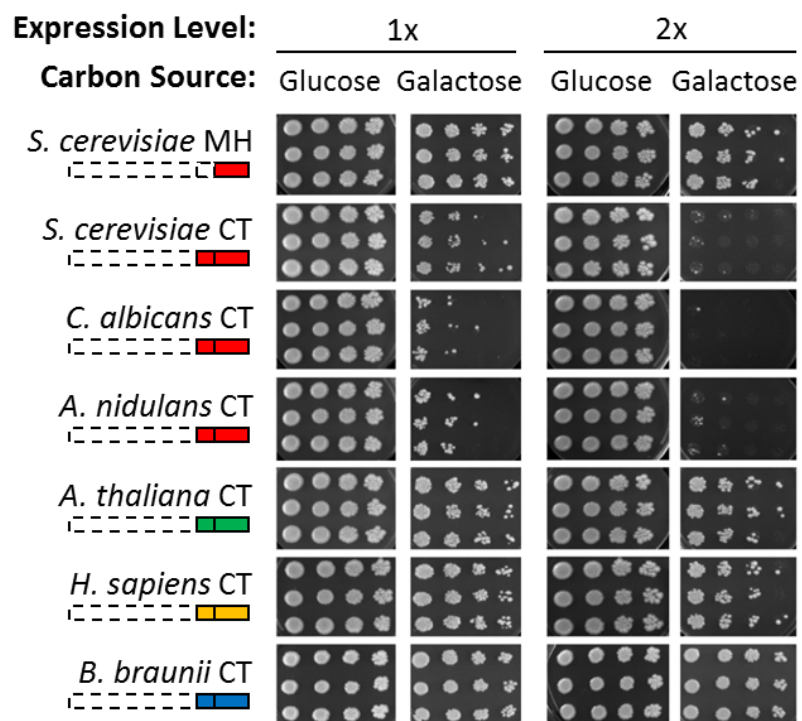


Figure 5.8: Fungal SQS carboxy-terminal domain expression inhibits wild-type yeast in a dose dependent manner. Yeast line BY4741 was transformed with expression vectors harboring either one (1x) or two (2x) copies of a SQS carboxy-terminal domain (without the catalytic domain signified by an empty box) from the yeast, *S. cerevisiae*, *C. albicans*, and *A. nidulans* (red); plant, *A. thaliana* (green); human, *H. sapiens* (yellow); or alga, *B. braunii* (blue). As a control, yeast were also transformed with constructs containing one or two copies of the *S. cerevisiae* membrane helix (MH) which does not include the hinge motif. Three independent transformants representing each construct were initially grown in glucose media, and then serially diluted and spot plated onto media where the promoter was either repressed (glucose) or induced (galactose).

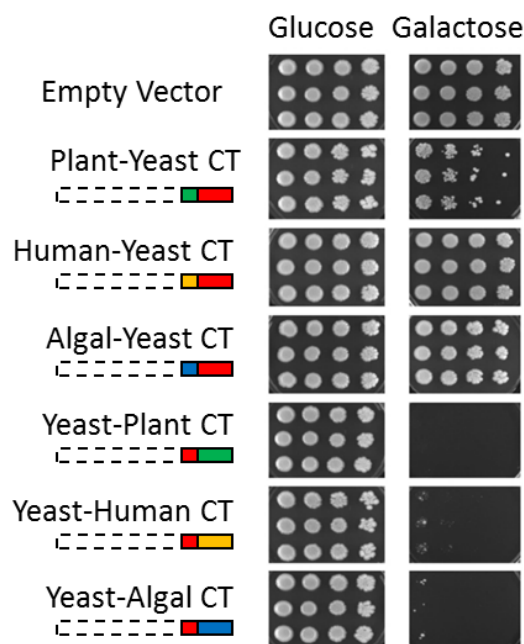


Figure 5.9: The fungal hinge motif is responsible for growth inhibition when expressing a SQS carboxy-terminal domain in wild-type yeast. Wild-type yeast line BY4741 was transformed with expression vectors harboring two copies of a chimeric SQS carboxy-terminal domain (without the catalytic domain signified by an empty box). Each chimeric C-terminal domain included a hinge motif or membrane spanning helix from a yeast, *S. cerevisiae* (red); plant, *A. thaliana* (green); human, *H. sapiens* (yellow); or alga, *B. braunii* (blue). Three independent transformants representing each construct were grown in glucose media, serially diluted five-fold and spot plated. Photographs were taken after 5 days of growth.

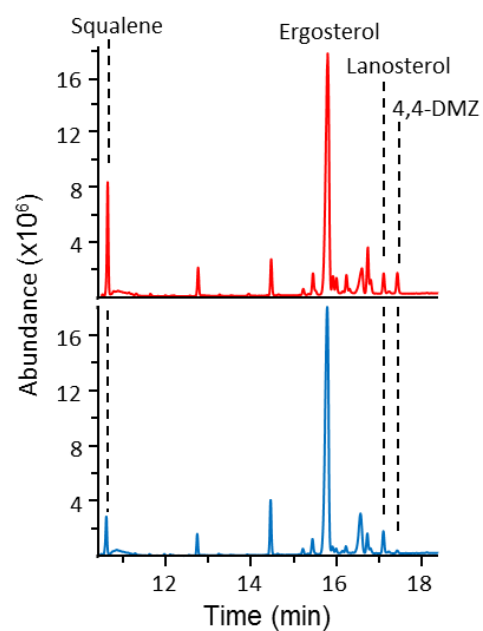


Figure 5.10: Profiling sterol accumulation following expression of a fungal C-terminal domain in wild-type yeast. Yeast line BY4741 was transformed with expression vectors harboring two (2x) copies of a SQS carboxy-terminal domain from a yeast, *S. cerevisiae* (red), or alga, *B. braunii* (blue). Cultures were grown in glucose media, induced for 3 days prior to extraction of non-saponifiable lipids. Extracted lipids were derivatized prior to GC-MS analysis.

solely caused by accumulation of a downstream sterol intermediate, then expression of the fungal C-terminal domain would not be expected to inhibit yeast growth. However, the same growth inhibition was seen in this yeast line as in the wild-type yeast BY4741 (Fig. 5.11).

Discussion

We have narrowed the region of the C-terminal domain responsible for modulating the toxicity phenotype associated with accumulation of the putative carboxysterol intermediates. This hinge motif appears to be serving some sort of structural role, perhaps helping to assemble the C4 demethylation complex in the downstream sterol pathway into an efficient, metabolic channel for ergosterol biosynthesis (Fig. 5.12). Disruption of this metabolic channel, especially at the level of the sterol C4-decarboxylase, may also lead to impacts on sphingolipid and/or fatty acid metabolism in the ER membrane [67,73,96].

This suggestion is corroborated by two additional observations. First, over-expression of the hinge domain with a membrane spanning helix from any fungi, but not from a plant or animal SQS, resulted in a very significant reduction in the growth rate of wild type yeast. Also, when we aligned the hinge domains from five fungal, five animal, five plant, and two algal SQS enzymes, a surprising degree of kingdom of life conservation and clustering is visible (Fig. 5.13). This has important implications. If the hinge domains did not provide for some important physiological function as demonstrated for the fungal hinge domains, one would not expect such sequence conservation to have developed and been maintained. Instead, if the hinge domains were only serving as linkers between the catalytic and membrane spanning domains, there would be much greater randomization much like the membrane spanning portion of these enzymes which retain hydrophobicity but not sequence identity within each kingdom.

The fact that this region is not only fungal-specific, but appears to be kingdom-specific offers equally distinct opportunities for technological applications. The fungal hinge domain does present itself as a target for antifungal therapeutic development, since our results demonstrate that interfering with the role of the hinge domain of SQS can lead to inhibition of fungal growth. This was described earlier when we co-expressed the active fungal SQS with an inactive fungal SQS, and here where we greatly over-expressed the C-terminal domain of a fungal SQS in wild-type yeast. Furthermore, we have demonstrated that only five amino acids of the fungal hinge motif need to be disrupted for yeast to manifest the toxic growth phenotype.

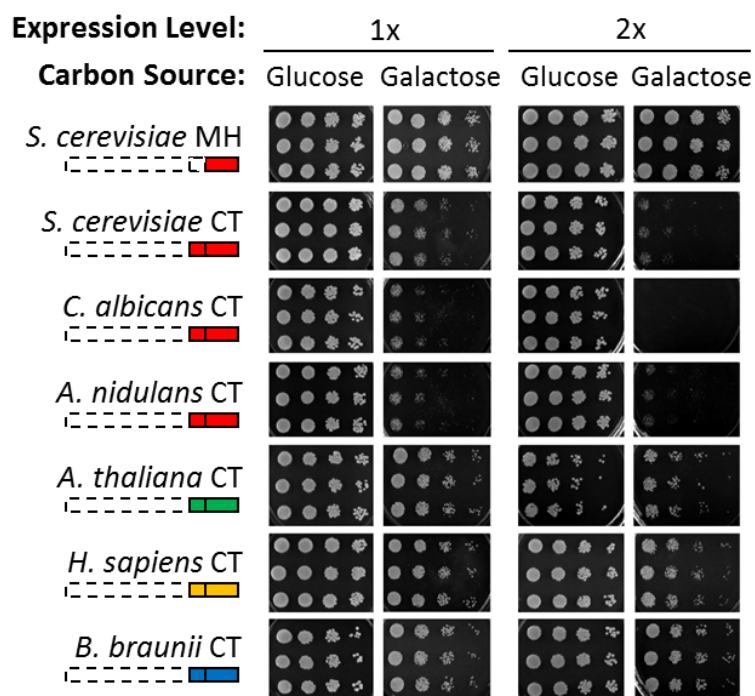


Figure 5.11: Fungal SQS carboxy-terminal domain expression inhibits yeast without a functional sterol biosynthetic pathway. Yeast line ZX178-08 ($\Delta erg9$ mutant) was transformed with expression vectors harboring either one (1x) or two (2x) copies of a SQS carboxy-terminal domain from fungi, *S. cerevisiae*, *C. albicans*, and *A. nidulans* (red); a plant, *A. thaliana* (green); a human, *H. sapiens* (yellow); or an alga, *B. braunii* (blue). Three independent transformants were spot plated on media where the promoter was either repressed (glucose) or induced (galactose) with exogenous ergosterol.

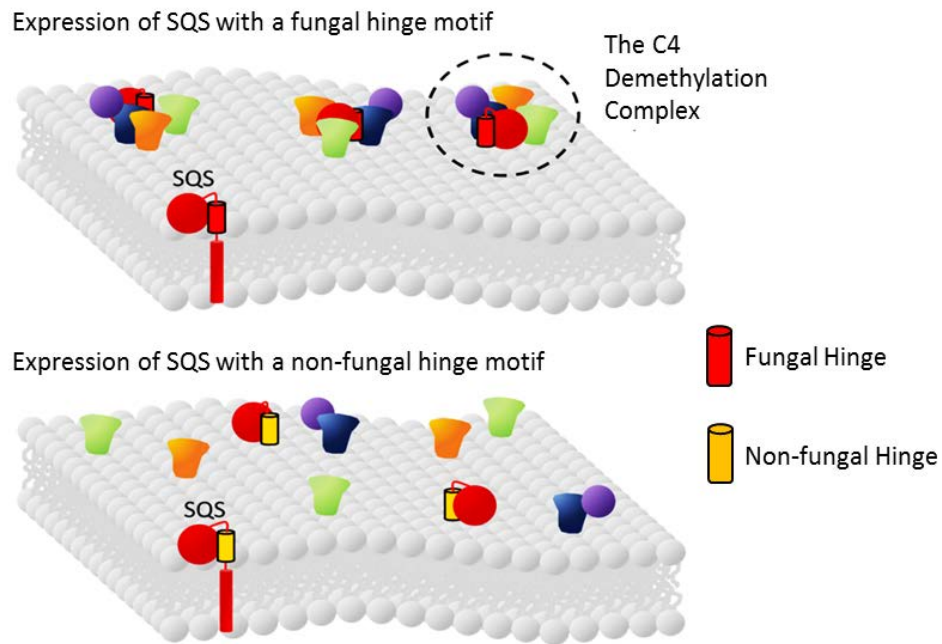


Figure 5.12: A conceptual model for how the organization of the sterol pathway might be facilitated by a non-catalytic hinge motif of SQS. Ergosterol biosynthesis is associated with the cytoplasmic face of the endoplasmic reticulum, and the efficiency of the C4 demethylation complex may be facilitated by the organization of enzyme complexes. The hinge motif of SQS would mediate the formation of these complexes, and expression of this enzyme with a hinge domain originating from a non-fungal SQS would not form such complexes, decreasing biosynthetic efficiency.

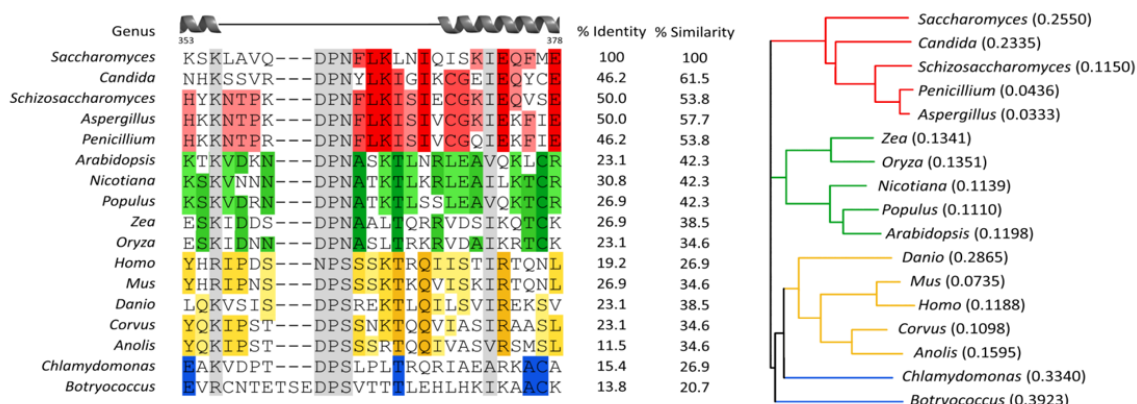


Figure 5.13: Comparison of the hinge motifs of squalene synthases across kingdoms of life. The hinge motif of *S. cerevisiae* (top line) was compared to the corresponding hinge motifs of four additional fungal SQS enzymes (red) as well as five plant (green), five animal (yellow) and two algal (blue) SQS enzymes. The structure above the alignment shows the predicted helix-loop-helix structure of the sequence as depicted in the published crystal structure of the human enzyme. Colored boxes show sequence identities for hinge motif amino acids within each kingdom, while gray shading illustrates conservation across all SQS enzymes. The percent sequence identity and similarity for each sequence compared to the *S. cerevisiae* hinge region is depicted to the right of the alignment beside a phylogenetic tree demonstrating the evolutionary relationship between these sequences.

This suggests that not only peptidomimetics but also small molecules could be used to affect a similar phenotypic outcome in pathogenic fungi. Much like the development of MDM2 inhibitors, which were modelled after a helical motif of the binding partner p53, the inhibitors could be modelled after the SKIEQ segment of the hinge motif and employed to inhibit the fungal C4-decarboxylase. Recent advances in the study of the parasites responsible for causing Leishmaniasis and Chagas disease in humans also show that controlling ergosterol biosynthesis is a promising method for antiprotozoal therapy, and an alignment of the hinge domain of SQS from these organisms suggests that this motif can also be utilized as a target for therapeutic development (Fig. 5.14). The critical role that this domain has in the sterol biosynthetic pathway of all eukaryotes further presents opportunities for the development of unique reagents to modulate sterol metabolism in both animals and plants.

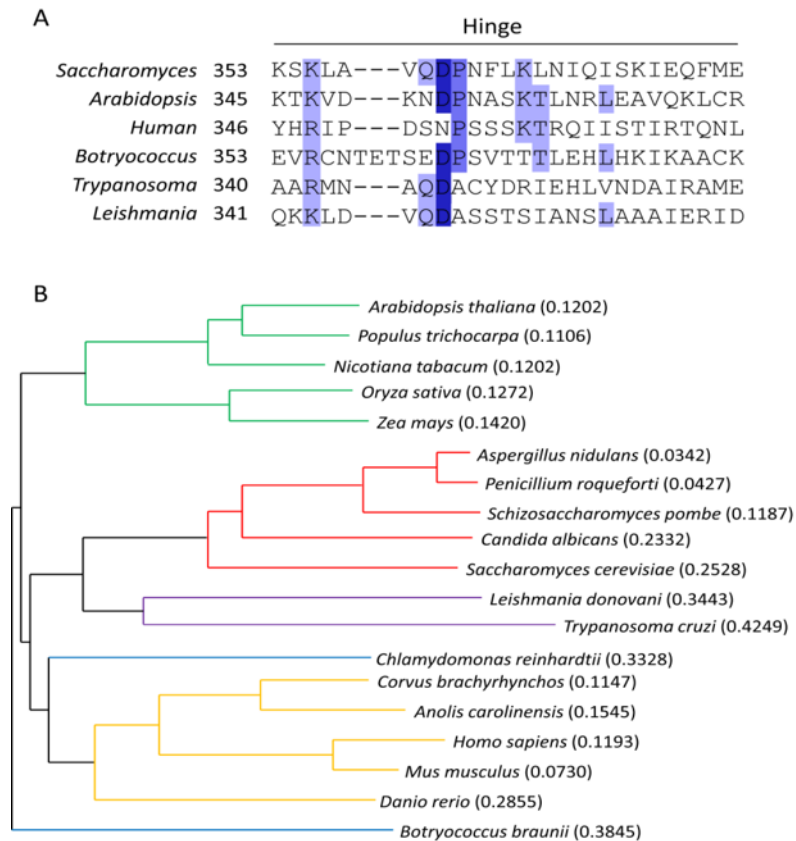


Figure 5.14: Alignment of hinge motifs from SQS enzymes in each kingdom of life including protozoans. (A) Alignment of the hinge motifs of SQS in each kingdom of life in comparison to the hinge motif of SQS from two protozoan parasites, *Leishmania donovani* and *Trypanosoma cruzi*. (B) A phylogenetic tree demonstrating the evolutionary relationship of the hinge motifs between multiple kingdoms of life including plants (green), fungi (red), vertebrates (yellow), algae (blue) and protozoans (purple).

Chapter 6: Additional Applications and Inferences: Investigating the Function of SQS Active Site Residues and Developing an Algorithm for Identifying Similar Motifs

Introduction

Not only do the findings in these studies present opportunities for the development of therapeutic agents, we also contend that the tools developed herein can be expanded to better understand the activity of SQS and related enzymes as well as a host of additional proteins conserved between kingdoms of life. While we have focused on a non-catalytic region of squalene synthase, the phenotype associated with the hinge motif can be applied to studies involving the enzyme's active site. Advances in our understanding of the structure-function relationship of amino acid residues in SQS catalytic activity have the potential to improve production platforms for triterpenes used as vaccine adjuvants, pharmaceuticals, nutraceuticals, cosmetics, and biofuels [97,98]. Important studies performed with yeast and human SQS proteins, as well as the dehydrosqualene synthase from *Staphylococcus aureus* (CrtM), have provided insight into the mechanism of SQS catalytic activity, focusing primarily on the aspartate-rich motifs in the five helical domains that make up the active site pocket [27,37,40,99]. The crystallographic studies using human SQS have also identified likely regions involved in NADPH binding, however this has not been determined empirically and there are many more aspects of this and similar enzymes that have not been mapped [37].

Initial studies in our laboratory interconverted residues from SQS in yeast with those in unique squalene synthase-like (SSL) enzymes found in algae to define which residues are involved in the first and second half reactions. SSL enzymes from the algae *B. braunii* race B produce squalene and botryococcene [33]. These enzymes utilize the same two step mechanism of SQS, but do so in multiple enzymes. SSL-1 catalyzes the first step, dimerizing the two molecules of FPP to produce PSPP. This PSPP is released from the enzyme and becomes available for two additional enzymes. SSL-2 uses PSPP and NADPH to produce 1'-1 linked squalene, while SSL-3 catalyzes a similar reaction but produces botryococcene with a 1'-3 linkage. This series of enzymes is distinct from the algal SQS which is also present in *B. braunii* and performs both the first and second half reactions for sterol biosynthesis [28]. The botryococcene oil associates with the extracellular matrix of the algae and is believed to provide

the buoyancy necessary to keep the algal colonies on the water's surface where sunlight is readily available.

Using reciprocal mutations in the active sites of these SSL enzymes, Bell et al. discovered a double mutant (N171A, G207Q) in SSL-3 which converted its product specificity from botryococcene to squalene [100]. Interestingly, the opposite mutations in the algal SQS (A177N, Q213G) did not lead to botryococcene production. These rational design approaches are made difficult by the low amino acid conservation between the SQS and SSL enzymes and would be improved by a screening platform able to select for squalene production from a pool of random mutant enzymes. Since we have shown that a non-fungal SQS is capable of complementing a SQS knockout mutation in yeast when the pGAL10 promoter is not induced, we asked whether a combination of SSL-1 and SSL-2 could also complement the knockout mutation. If so, then we supposed that SSL-3 mutants could be screened for their ability to produce squalene by selection on SC glucose media. The work described below surrounding this effort was done in collaboration with Dr. Stephen Bell who was also a graduate student in the laboratory of Dr. Joe Chappell.

The findings related to the hinge motif of SQS also have broader implications for other conserved proteins involved in cell growth and metabolism. We have presented evidence for the discovery of a protein motif associated with a highly conserved enzyme in eukaryotes that is distant from the catalytic site and yet is essential for genetic complementation. We have further demonstrated that this motif is conserved within but not between kingdoms of life, that the hinge motif from plants and animals cannot replace the hinge motif of a fungal SQS. We suggest that there are similar protein motifs associated with other enzymes that are kingdom of life specific and play essential physiological roles in eukaryotic cells.

Identifying these kingdom-specific motifs or domains in other proteins presents an interesting challenge. Current protein databases search for protein sequences using functional domains such as active sites that are conserved not only within kingdoms of life, but between kingdoms as well. Sequences such as the hinge motif that are conserved within but not between kingdoms show less conservation overall and are therefore overlooked. Methods have been described that search for specialized functions in protein sub-families, such as ligand binding and protein-protein interactions, using protein sequence alignments. Some of these methods, including SDPpred [101], ProustII [102], and Multi-harmony [103] allow the user to define the groups for analysis, and so can be used to look at proteins within kingdoms of life

rather than protein sub-families. However, many of these methods have been shown to have modest and varying success identifying these sites. In order to identify kingdom-specific motifs, we have designed a preliminary algorithm which compares amino acid conservation within verses across kingdoms of life and have validated the algorithm's ability to identify the SQS hinge motif. The work designing the algorithms to examine kingdom of life specific domains was done in collaboration with Dr. Jinze Liu's laboratory in the Department of Computer Science, at the University of Kentucky and involved Corrine Elliott (undergraduate) and Satrio Husodo (post-back student).

Results

Determining a complementation phenotype for SSL1-SSL3 constructs in $\Delta erg9$ yeast

In order to enhance our understanding of the active site residues necessary to produce squalene from FPP, we designed a library screening method to identify mutations able to convert the product specificity of SSL-3 from botryococcene to squalene. Previous attempts to express the SSL-1 gene in a $\Delta erg9$ yeast line led to growth inhibition, suggesting that the overproduction of PSPP or PSOH in yeast does lead to growth inhibition. Co-expression of SSL-1 and SSL-2 separately also led to growth inhibition, unlike expression of SSL-1 and the partially inactive yeast and algal SQS enzymes described in chapter 3. This suggests that the substrate affinity of SSL-2 may not be high enough to efficiently convert PSPP into squalene.

Given these findings, it is likely that attempts to mutate SSL-3 would also lead to poor substrate affinity. Therefore, we used a version of SSL-1 linked to SSL-3 using a GGSGx3 linker sequence (SSL1-SSL3) [49]. Using the ZXB yeast line ($\Delta erg9,1$), we first expressed a variety of constructs harboring the *SSL1-SSL3* linked gene, as well as a version with the N171A and G207Q mutations (*SSL1-mtSSL3*) already known to convert product specificity with and without the yeast C-terminal domain (YCT). The C-terminal domain was added for two reasons. We proposed first that adding a membrane spanning helix would help localize the enzyme and increase its access to the substrate FPP, and second that it would allow the squalene producing version of this dual enzyme to fully complement the *SQS* knockout mutation in yeast. Cultures representing three colonies from each transformation were used to inoculate cultures which were grown at room temperature for 7 days to quantify the levels of botryococcene and squalene produced (Fig. 6.1A). Expression of each of the *SSL1-SSL3* genes in a yeast line that is not able to use squalene to produce sterols (ZXB) led to the identification of both botryococcene

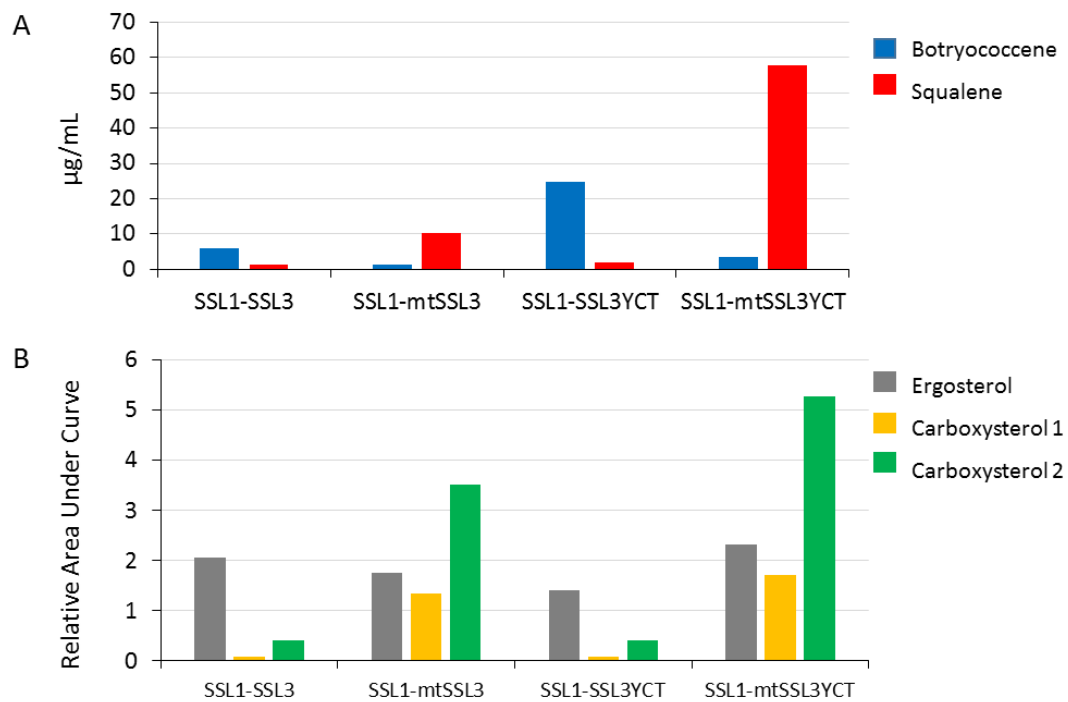


Figure 6.1: The SSL1-mtSSL3YCT construct produces increased squalene in yeast which feeds into the sterol biosynthetic pathway. A) Expression vectors containing the *SSL1-SSL3* gene with or without the mutations N171A and G207Q (mt) and with or without the C-terminal domain of *S. cerevisiae* SQS (YCT) were transformed into the ZXB yeast line ($\Delta erg9,1$). Cultures were grown for 7 days at room temperature before they were extracted and triterpenes quantified by GC-MS. B) The same *SSL1-SSL3* constructs were transformed into yeast line ZX178-08 ($\Delta erg9$), and cultures were induced for 3 days before ergosterol and carboxysterol intermediates were quantified.

and squalene by GC-MS, however the level of each product depended on which variation of this gene was expressed. The SSL1-SSL3 protein without the two amino acid mutations in SSL-3 or the yeast C-terminal domain produced primarily botryococcene (7 µg/mL) with less than 2 µg/mL of squalene. Adding the two point mutations in the SSL-3 gene converted the product profile, leading to approximately 10 µg/mL of squalene and less than 2 µg/mL of botryococcene. The addition of the yeast SQS C-terminal domain clearly improved product accumulation, leading to 25 µg/mL botryococcene with the SSL1-SSL3YCT protein and almost 60 µg/mL of squalene when the *SSL-3* gene was mutated. This is not surprising since it has previously been demonstrated that expression of truncated forms of SQS led to very low product levels, suggesting that ER membrane localization is critical for efficient use of the substrate FPP [39].

We then transformed each of the four constructs into the ZX178-08 yeast line (*Δerg9*) to analyze yeast growth when squalene was allowed to continue through the sterol biosynthetic pathway. Colonies from each transformation were grown in SCE glucose and induced for 3 days in SCE galactose media prior to GC-MS analysis of the derivatized lipids (Fig. 6.1B). Levels of both ergosterol and carboxysterols were assessed using a selective ion monitoring at $m/z = 468$. The levels of ergosterol stayed relatively steady independent of the gene being expressed, while the level of carboxysterols increased along with changes in squalene production. Expression of the mutated *SSL-3* genes led to the highest levels of carboxysterol intermediates, and the addition of the yeast C-terminal domain did not decrease accumulation of this toxic intermediate. This suggests that while the hinge motif in the C-terminal domain of SQS is critical for preventing carboxysterol accumulation in the context of a full-length SQS, attaching the domain to another enzyme may disrupt the structure of the hinge motif, preventing it from assisting the C4 demethylation complex.

While the addition of the yeast C-terminal domain to SSL1-SSL3 did not decrease the level of the toxic downstream intermediates, the presence of a membrane spanning helix did increase the overall production of both squalene and botryococcene. For this reason, we continued to use the genes containing this domain and proceeded to analyze yeast growth using spot plate analysis. Three colonies from each transformation were grown in SCE glucose, 5x serial diluted and spotted on both glucose and galactose media with and without exogenous ergosterol (Fig. 6.2). As expected, yeast expressing the SSL1-SSL3YCT, which primarily makes botryococcene, were only able to grow on media containing exogenous ergosterol, and growth was not inhibited by induced gene expression. However, expressing the same gene with a

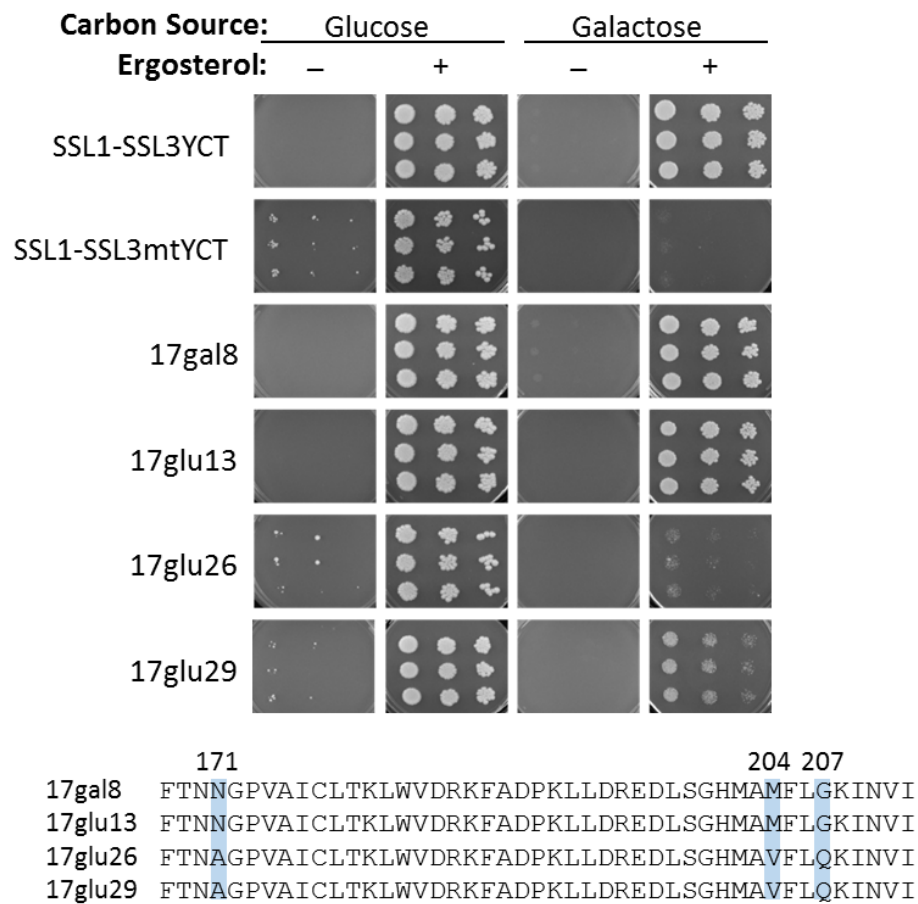


Figure 6.2: Expression of algal SQS genes produced by library mutagenesis and identified by screening for the non-fungal SQS growth phenotype. Expression constructs containing SSL1-SSL3YCT (wild type) or mutated at positions N171, M204, and G207 were transformed into yeast line ZX178-08 ($\Delta erg9$). Transformants were grown in non-inducing media (SCE glucose) for five days before three independent transformants were spot plated onto non-inducing and inducing media with or without exogenous ergosterol.

mutated SSL-3 led to impaired growth on glucose media without exogenous ergosterol, and growth was fully inhibited once the promoter was induced by galactose, a phenotype identical to expression of a full-length non-fungal SQS.

Library screening identifies mutations capable of converting the catalytic specificity of SSL3

Since there was a clear difference in the growth phenotype of yeast expressing mutated and non-mutated SSL1-SSL3 enzymes, we next produced a library of mutations in SSL-3 involving amino acid positions 171 and 207. If this selection platform was successful, we expected to uncover mutations capable of converting SSL1-SSL3YCT to a squalene synthase and to recover the known mutant gene *SSL1-mtSSL3YCT* with N171A and G207Q mutations. Primers were designed for saturation mutagenesis, with the ability to produce a total of 400 possible mutants. In order to ensure that we were successfully mutating these positions to all possible amino acids, 47 random constructs from the library were purified from *E. coli* and sequenced. We discovered that 75% were mutated at one or both of the targeted positions, and the frequency of substitution was 5-15% for each possible amino acid. We then transformed the library into ZX178-08 yeast and selected for mutants on media that either repressed (SC glucose) or induced (SC galactose) the promoter without any exogenous ergosterol.

Forty independent transformants were collected from the selection plates and the plasmids responsible for the partial complementation phenotype were isolated and sequenced. Two of the isolated constructs harbored SSL1-SSL3YCT genes without any mutations. One of these was from an SC galactose plate (17gal8) and another from SC glucose (17glu13) while two were found to contain the expected mutations at amino acid positions 171 and 207 (17glu26 and 17glu29). These four constructs were transformed back into yeast line ZX178-08 and analyzed using spot plating on all four media types, SC glucose and SC galactose with and without exogenous ergosterol (Fig. 6.2). Other mutations uncovered by this analysis are listed in Table 6.1.

As expected, the constructs containing the chimeric gene without any additional SSL-3 mutations were able to grow on media with exogenous ergosterol independent of the carbon source. Interestingly, the two mutated constructs were able to grow on three of the four media types. As expected, growth was robust on SCE glucose media, and greatly impaired on the same media without added ergosterol. However, these transformed yeast were able to grow on galactose media with added ergosterol. A closer look at the sequencing data from these

Table 6.1: Independent clones recovered from the SSL1-SSL3YCT mutant library on SC glucose and SC galactose solid media. ZX178-08 competent cells were transformed with the mutant library, and transformed yeast were allowed to recover in YPDE media for 18 hours and rinsed with sterile water prior to plating on both SC glucose and SC galactose solid plates. Colonies grown within 15 days were isolated and the DNA sequenced.

Mutations	# Independent Clones	Selection Media
N171,G207 (wt)	2	SC galactose
N171A, M204V, G207Q	2	SC glucose
N171A,G207E	1	SC glucose
N171S,G207Q	13	SC glucose
N171S,G207E	5	1 on SC galactose 4 on SC glucose
N171T,G207Q	1	SC glucose
N171T,G207E	9	3 on SC galactose 6 on SC glucose
N171C,G207Q	7	4 on SC galactose 3 on SC glucose

constructs revealed an additional M204V mutation which could be contributing to this phenotype.

Chemical profiling of these four enzymes showed that the two non-mutated constructs made botryococcene at expected levels, while 17glu26 and 17glu29 produced mostly squalene (Fig. 6.3). 17glu29 produced the same levels of squalene and botryococcene as SSL1-mtSSL3YCT, which was surprising since yeast expressing this enzyme were able to grow on SCE galactose media. This led us to suspect that the amount of squalene entering the sterol pathway was decreased. On the other hand, the 17glu26 construct produced half the amount of squalene when compared to the SSL1-mtSSL3YCT control. If this was due exclusively to the additional M204V mutation, then the same would have been true for 17glu29. Therefore, there may be an additional mutation in the backbone of the expression vector of 17glu26 that changes the expression level of this enzyme and so decreases the amount of squalene accumulating.

Developing a naïve algorithm able to highlight the hinge motif of squalene synthase

The findings related to the hinge motif may have broader implications which extend beyond the sterol biosynthetic pathway. The hinge motif uncovered when mapping the SQS complementation phenotype is highly conserved within each kingdom of life, but not between Kingdoms. It is likely that this pattern can be identified in other proteins involved in cell growth and metabolism that are found in all eukaryotic cells. Hence, we designed an algorithmic approach to identify these motifs on a broader scale, using SQS as an initial test case.

We first used the existing T-Coffee program [104] to align five characterized SQS protein sequences from each kingdom of life: plant, animal, and fungal. In order to identify kingdom-specific regions of the alignment, each position was scored based on two conditions. The first condition involved conservation within each individual kingdom of life (\hat{C}_k). For instance, if the second position in the alignment for the fungal SQS sequences is predominantly glycine, and it is present in four of five sequences, the fungal identity score for that position would be 0.8 (4/5). The second condition highly scored the positions in the alignment with amino acids that are highly conserved throughout all kingdoms of life (\hat{C}_{all}), such as the initial methionine or those in the active site. These positions would have a score of one (15/15). The second position consists of nine glycine residues in 15 total sequences for a score of 0.6 (9/15). These two values, within the fungal kingdom and between kingdoms, are then used to calculate fungal-specificity by subtracting the all-kingdom identity value from the fungal-specific identity value (0.8-0.6).

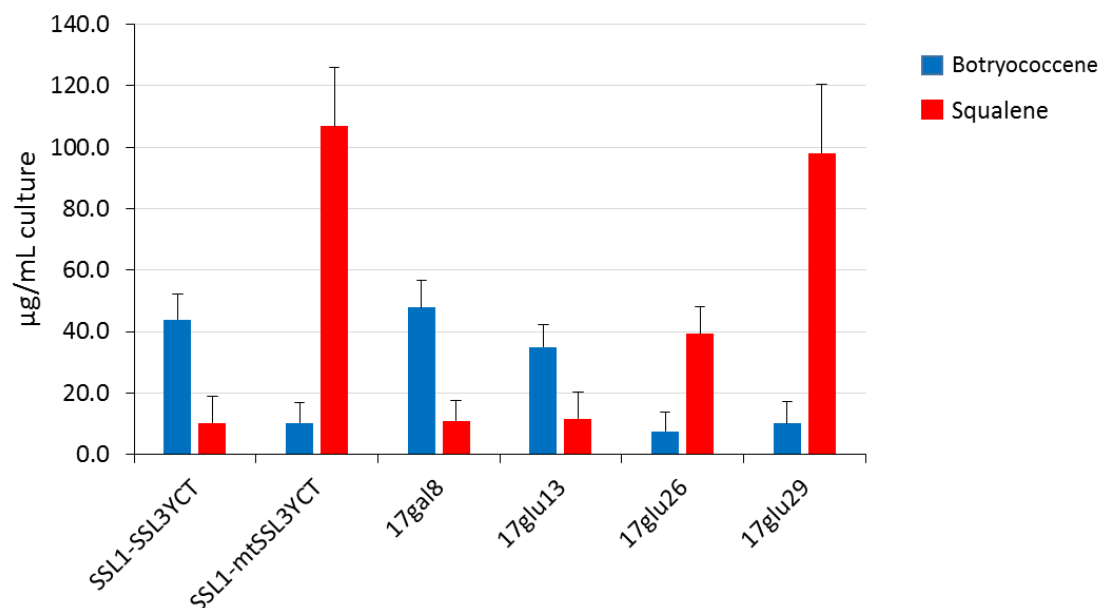


Figure 6.3: The 17glu26 and 17glu29 mutants have a converted product specificity but produce different levels of squalene. Expression vectors recovered from library mutagenesis containing the *SSL1-SSL3YCT* gene without (17gal8 and 17glu13) or with the mutations N171A, M204V, and G207Q (17glu26 and 17glu29) were transformed into the ZXB yeast line ($\Delta erg9,1$). Starter cultures grown in SCE glucose were used to induce 3 mL of liquid SCE galactose media. Cultures were grown for 7 days at room temperature before they were extracted and triterpenes quantified by GC-MS.

Kingdom-specificity (S) is then defined as the sum of the specificity values assigned to each individual kingdom (\hat{S}_k) and the scores are normalized to a value between 0 and 1 (Fig. 6.4A).

$$S = \prod_k \hat{S}_k = \prod_k \left\| \hat{C}_k - \hat{C}_{all} \right\|$$

The SQS hinge motif does not consist of a single or even a pair of highly conserved amino acids, but rather a series of 16 highly conserved and closely grouped amino acids folded into a 3-dimensional structure. We therefore implemented a sliding window to highlight motifs rather than highly conserved but individual amino acids within the alignment (Fig. 6.4B). The sliding window considers each position in the alignment and adjusts the score depending on the scores of the surrounding positions. More specifically, the score at each position becomes the average of the score at that position and four positions in each direction. This takes into account amino acids with R groups that would be in close proximity if the structure of the motif is an alpha helix with the expected 3.6 amino acids per turn. The hinge motif then becomes the second highest scoring sequence within the fungal SQS protein. Mapping the amino acid positions that have a kingdom-specificity score higher than 0.3 to the homology model of *S. cerevisiae* SQS highlights those motifs that are available for protein-protein interactions (Fig. 6.4C).

The results from this naïve algorithm were then compared to the available functionality predicting programs Proust II, SDPpred, and multi-harmony. The Proust II server was only able to identify a single amino acid position near the amino-terminus of SQS with a Z score greater than 2.5. Results from SDPpred and the two algorithms that are associated with multi-harmony are depicted in figure 6.5. SDPpred results were converted into positive values for a more direct comparison to results from the other programs. While each of these algorithms does identify the SQS hinge motif, it is not as clearly defined as the naïve algorithm.

Discussion

The active sites of squalene synthase and related triterpene synthases have been difficult to study in part due to the two step reaction mechanism. The discovery of the squalene synthase-like enzymes in *B. braunii* provided a new method for exploring the requirements for each step independently. Using these SSL enzymes, we sought to develop a screening method for investigating the effect of multiple amino acid mutations on squalene production.

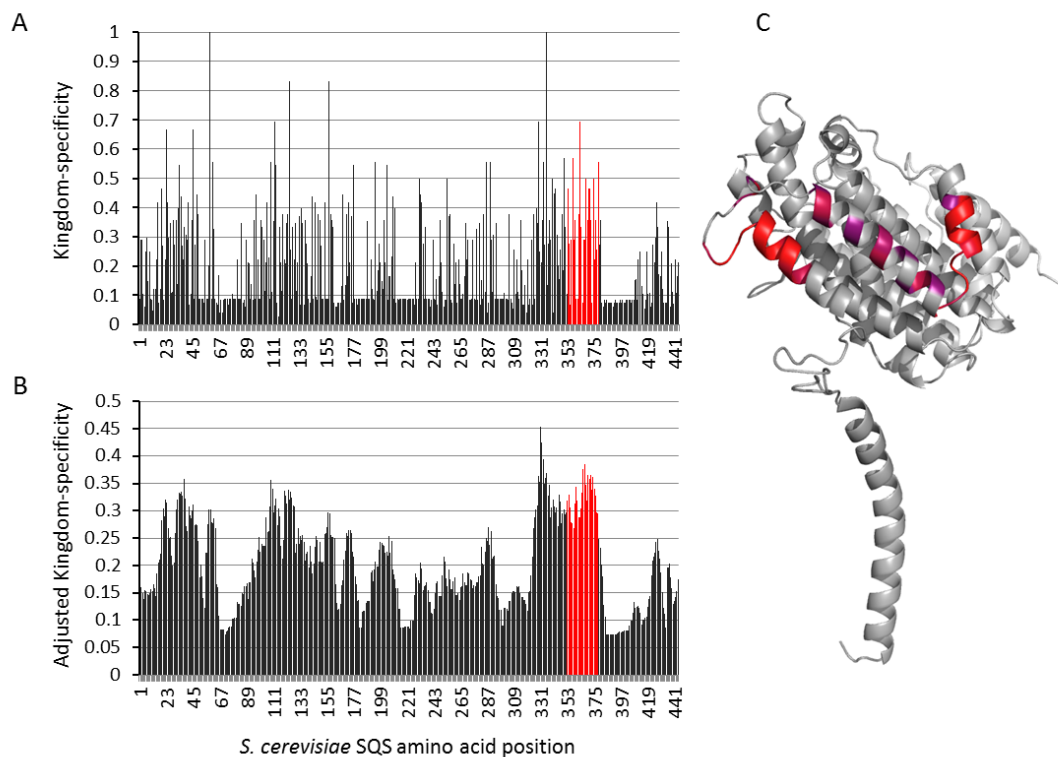


Figure 6.4: The naïve algorithm for identifying kingdom-specific motifs identifies the hinge motif of SQS. (A) An alignment of 5 fungal, 5 plant, and 5 animal SQS proteins was analyzed using the naïve kingdom of life analysis (KoLa) algorithm and the resulting kingdom-specificity measures are graphed along the amino acid sequence from *S. cerevisiae* SQS. The hinge motif is shown in red. (B) A sliding window was then implemented to add a weighted score to identify motifs with high kingdom-specificity. (C) An *S. cerevisiae* SQS homology model was colored according to the scores from the Kola algorithm with a sliding window. Amino acid positions with an adjusted score of 0.35 were colored red, while scores greater than 0.3 were colored purple.

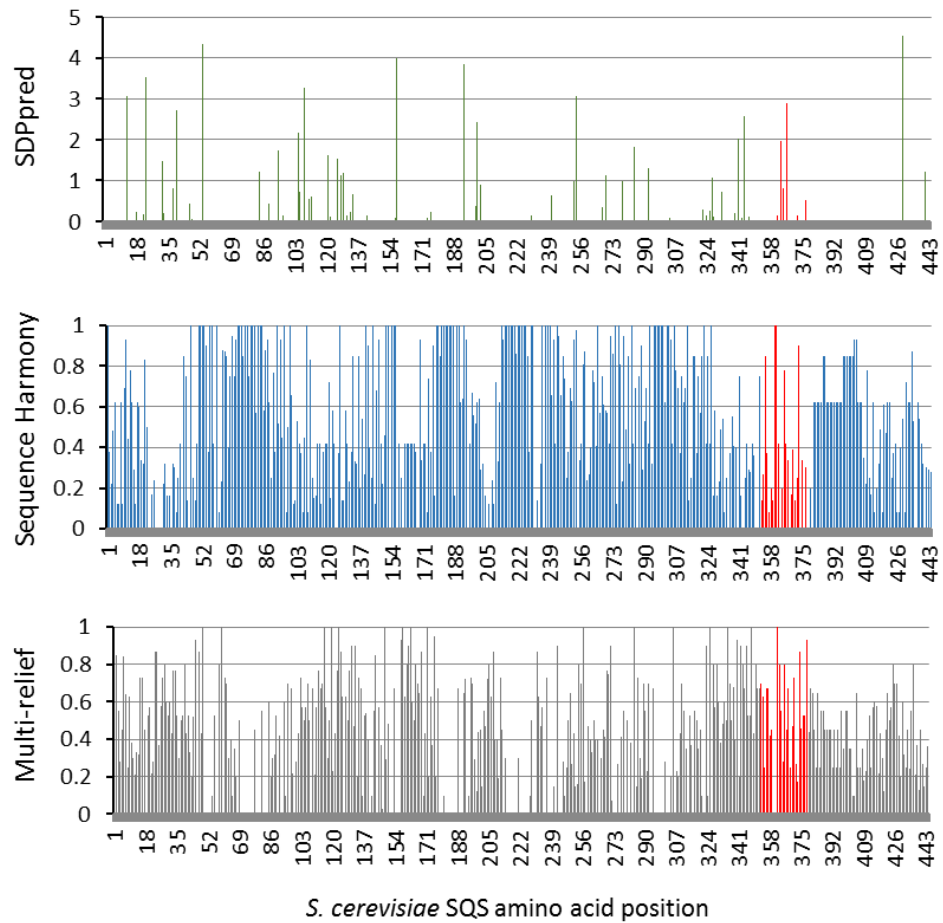


Figure 6.5: Currently available algorithms do not clearly identify the SQS hinge motif. The alignment of 5 fungal, 5 plant, and 5 animal SQS proteins was analyzed using the SDPpred and Multi-harmony (Sequence Harmony and Multi-relief) servers and the resulting functionality scores graphed for comparison to the naïve KoLa algorithm. Amino acids corresponding to the hinge motif are colored red.

Our results show that the asparagine at position 171 can be replaced by a less polar residue of similar size as long as the glycine at position 207 is mutated to a charged residue. The difference between the production of botryococcene and squalene lies in the way the cyclopropyl intermediate PSPP is opened, leading to either a 1-1' or 1-3' linkage. Since the two sites we chose for saturation mutagenesis, an asparagine at position 171 and glycine at position 207, are involved in specifying one of these two products, they are likely responsible for determining how the cyclopropyl ring is oriented in the active site pocket. Molecular modeling of these two enzymes based on the crystalized human SQS [100] shows that these residues may be located opposite each other with their R groups oriented into the active site.

The presence of an additional M174V mutation in the two genes resembling SSL1-mtSSL3YCT recovered from the library was unexpected. Since the C-terminal domain of the yeast SQS did not allow the chimeric SSL gene to fully complement the SQS genetic deficiency in this yeast line, leading to carboxysterol accumulation, there was an additional pressure on this enzyme other than the need to produce squalene for ergosterol biosynthesis. For instance, any mutations that would make the enzyme less stable, or more easily regulated, would be preferred as the amount of squalene entering the downstream pathway and accumulating as toxic intermediates could be more easily controlled.

While the growth phenotype caused by expression of a SQS without a functioning fungal hinge motif has allowed us to begin investigating the role of active site residues in squalene biosynthesis, the knowledge gained from discovery of the hinge motif led to the development of a method for identifying additional kingdom-specific motifs. What we have presented here is only an initial proof of principle, and many challenges remain before this approach is able to be used on a larger scale. For instance, there must first be a method to identify proteins that are present and highly conserved in many kingdoms of life and collect grouped sequences that can be aligned. The alignment algorithm that best supports the identification of kingdom-specific motifs must be determined, and even then dramatic changes in protein sequences within a kingdom of life have the potential to disrupt the overall alignment. For this reason we plan on identifying a method to remove sequences that act as outliers in the alignment and score the alignments based on amino acid similarity as well as identity. Once kingdom-specificity can be determined using a linear protein sequence, we must also consider protein three-dimensionality and the formation of kingdom-specific domains from protein folding.

Once developed, we believe that this algorithmic approach will shed light on evolutionary mechanisms regulating many critical cellular metabolic pathways and aid both in the generation of new therapeutics to control diseases and the biosynthesis of additional high-value industrial products.

Chapter 7: Concluding Remarks

This dissertation, explored the non-catalytic function of the C-terminal domain of SQS in the sterol biosynthetic pathway. Beginning with a detailed description of the growth phenotype caused by expression of fungal and non-fungal SQS proteins in a $\Delta erg9$ yeast line (Chapter 3), we discovered a likely cause of growth inhibition (Chapter 4) and mapped the motif in the C-terminal domain of SQS responsible for the phenotype (Chapter 5) that will aide in the future development of a screening method for identifying novel antifungal agents. Highlights of Chapter 3 include the observation that *SQS* genes from each kingdom of life were indeed able to complement a *SQS* genetic deficiency, although growth was impaired when the *SQS* was non-fungal. Overexpression of a *SQS* with catalytic activity but with a non-fungal C-terminal domain led to complete growth inhibition. This was despite the finding that all *SQS* enzymes were expressed and active in yeast, and complementation or the lack thereof did not correlate with levels of squalene production *in vivo*. This phenotype, though it occurred through a molecular mechanism distinct from *SQS* catalytic activity, was dependent on a functioning sterol pathway. In Chapter 4, this growth toxicity was mapped to the disruption of C4 demethylation, and more specifically a step involving sterol C4-decarboxylation (SDC), by manipulating carbon flux and profiling sterols within total lipid extractions.

There are a number of directions in which this work could next be pursued. Our work, as well as previous investigations with various *SQS* proteins, showed that this growth phenotype can be described as fungal versus non-fungal. However, complementation of the *SQS* genetic deficiency has only been described for five fungal *SQS* proteins, and the lack of complementation with a limited number of non-fungal *SQS* proteins. Further work would need to be completed with *SQS* genes from a much wider variety of organisms to be confident in the use of these terms. The specific cause of growth inhibition caused by a missing fungal hinge motif also requires more investigation. While we are here tentatively identifying the two peaks identified in yeast expressing a non-fungal *SQS* as carboxysterol epimers, these sterols must be purified and NMR studies performed to verify the identity of the accumulating sterols definitively. Addition of the carboxysterol to yeast lines able to take up exogenous sterols would be expected to inhibit growth as well. It is also unclear whether the *SQS* hinge motif interacts directly with the C4-decarboxylase or disrupts activity of the C4-decarboxylase through an indirect mechanism in the sterol biosynthetic complex.

The results in Chapter 5 return from the downstream sterol pathway to the C-terminal domain of SQS and identify the hinge motif responsible for mediating complementation of the SQS knockout mutation. It is unclear why point mutations in this hinge motif do not seem to disrupt its ability to rescue C4-decarboxylase activity in yeast but can only confer the complementation phenotype to a non-fungal SQS if the entire motif is present. Combinations of point mutations involving isosteric and isoelectronic substitutions will be required to explore the function of individual residues. Perhaps most interesting, however, is the finding that this motif is not only fungal-specific, but may be kingdom specific as well. We are not suggesting that this motif has the same function in each kingdom of life, but rather that it has a defined function in each kingdom which creates evolutionary pressure leading to sequence conservation over time. Additional experiments in plant and human cells are required to confirm that this claim is accurate. Using the tools and findings in the previous chapters, we have begun the process of creating screening protocols and algorithms for characterizing the function of residues in the active site of SQS and identifying a variety of hinge-like kingdom-specific motifs.

Many of the findings described throughout this dissertation lead us back to the development of a novel and promising antifungal agent based on the hinge motif of a fungal SQS. There are a multitude of characteristics that must be considered when identifying a target that has potential for the production of an anti-infective agent. For instance, it must be able to function against medically important pathogens. While most of our work involved the growth of nonpathogenic brewer's yeast, we have also confirmed that SQS proteins from the two most relevant genera of pathogenic fungi, *Candida* and *Aspergillus*, have hinge motifs that are capable of functioning in the same manner as the motif found in *S. cerevisiae* SQS. It is also critical that any novel therapeutic has a mechanism that is broad spectrum among all similar infectious organisms without causing undue toxicity in the host. The fungal-specificity, and perhaps even kingdom-specificity of the hinge motif identified here suggests that this would be the case for therapeutics designed according to this motif. A newly developed anti-infective agent would also need to inhibit the growth of pathogens that have become resistant to existing drugs. Since the most commonly administered antifungal agents, the azoles, function by the development of a side-pathway in ergosterol biosynthesis that includes the C4 demethylation complex, inhibition of a step in this complex would be beneficial. An inhibitor that is designed to mimic the hinge motif of a fungal SQS and disrupt the downstream sterol C4-decarboxylase has the potential to be a safe and specific inhibitor of fungal growth.

APPENDIX A: Abbreviations

BY4741 – Wild-type haploid yeast line

C-terminal – Carboxy-terminal

ER – Endoplasmic reticulum

FPP – Farnesyl diphosphate

GC-MS – Gas Chromatography-Mass Spectrometry

NADPH – Nicotinamide adenine dinucleotide phosphate

N-terminal – Amino-terminal

PSOH – Presqualene alcohol

PSPP – Presqualene diphosphate

SC – Synthetic complete media

SCE – Synthetic complete media with exogenous ergosterol

SDC – Sterol C4-decarboxylase

SKR – Sterol C3-ketoreductase

SMO – Sterol C4-methyloxidase

SSL-1 – Squalene synthase-like enzyme from *B. braunii* that converts FPP into PSPP

SSL-2 – Squalene synthase-like enzyme from *B. braunii* that converts PSPP into squalene

SSL-3 – Squalene synthase-like enzyme from *B. braunii* that converts PSPP into botryococcene

SQS – Squalene synthase

YCT – Yeast squalene synthase C-terminal domain

ZX178-08 – Yeast line capable of exogenous sterol uptake with a $\Delta erg9$ mutation

ZXB – Yeast line capable of exogenous sterol uptake with $\Delta erg9$ and $\Delta erg1$ mutations

ZXE – Yeast line capable of exogenous sterol uptake with $\Delta erg9$ and $\Delta erg7$ mutations

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